Effect of cyclosporine A and tacrolimus on OATP1B1 mediated transport of atorvastatin in an *in vitro* whole cell model

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Behnaz Zabihyan

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ABBREVIATIONS

Abbreviation	Full name
ABC	ATP-binding cassette
ATP	Adenosine-5'-triphosphate
BCRP	Breast cancer resistance protein
Bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CHD	Coronary heart diseases
CsA	Cyclosporine A
CYP	Cytochrome P450
DHEAS	Dehydroepiandrosterone sulphate
DMEM	Dulbecco's modified eagles medium
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
FBS	Foetal bovine serum
FKBP-12	FK506-binding protein-12
HBSS	Hanks' Balanced Salt Solution
HDL	High density lipoprotein
HEK293	Human embryonic kidney 293 cells
HLA	Human leukocytes antigen
HMG-CoA	3-Hydroxy-3-methylglutaryl Coenzyme A
HPLC	High-performance liquid chromatography

Abbreviation	Full name
IC_{50}	Inhibitory concentration 50%
IL_2	Interleukin 2
IS	Internal standard
K_i	Inhibitory constant
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
LTC_4	Leukotriene C_4
MRP	Multidrug resistance associated protein
MS	Mass spectrometry
MRM	Multiple reaction monitoring
NFAT	Nuclear factor of activated T cells
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
OCTN	Organic cation/carnitine transporter
PCR	Polymerase chain reaction
PGE_2	Prostaglandine E_2
P-gp	P-glycoprotein
RT	Reverse transcriptase
SLC	Solute carrier
T3	Triiodothyronine
T4	Thyroxine
Tac	Tacrolimus
UTG	Uridine 5'-diphospho-glucuronosyltransferase

ABSTRACT

BACKGROUND: Dyslipidaemia is more frequent in solid organ transplant recipients than in the general population. In order to reduce the overall cardiovascular risk in transplant patients lipid-lowering drugs, especially statins, have become widely used. However, the use of statins in transplant recipients taking cyclosporine A (CsA) results in significantly increased risk for statin induced side-effects such as myopathy and also rhabdomyolysis. Statin plasma concentrations are also increased several-fold and may be responsible for this increased risk. The other calcineurin inhibitor, tacrolimus (Tac), does not present such an effect on statin plasma concentrations during ordinary clinical use.

AIM AND METHOD: The present study elucidated the *in vitro* cellular aspects and the mechanism of the pharmacokinetic interaction between atorvastatin (HMg-CoA reductase inhibitor) and the two calcineurin inhibitors, CsA and Tac. The inhibitory effect of CsA and Tac on cellular uptake of atorvastatin via the organic anion transporter polypeptide 1B1 (OATP1B1) was investigated in an *in vitro* over expression whole cell model.

RESULTS: Atorvastatin was shown to be transported via the OATP1B1 transporter since cellular uptake was higher in OATP1B1 transfected HEK293 cells compared to mock cells. Inhibition of cellular uptake of atorvastatin was observed with CsA in OATP1B1 transfected cells. No clear inhibition of cellular uptake of atorvastatin was however observed for Tac in the OATP1B1 model, at least not in the lower concentration range investigated.

CONCLUSION: The present *in vitro* study indicates that the cellular uptake of atorvastatin is mediated by OATP1B1 transporters and that CsA inhibits this drug transport. Tac on the other hand did not interfere with cellular uptake of atorvastatin, at least not in the concentration range investigated in this thesis. The observed interaction between atorvastatin and CsA is in line with findings in previous clinical studies, however further investigations is necessary in order to further characterize these findings.

1. INTRODUCTION

1.1 Renal transplantation

Kidney transplantation is the most commonly performed transplant procedure, and has a one year graft survival of almost 90% for diseased donors. Diabetes, hypertention, and chronic pyelonephritis are the most common diseases leading to kidney transplantation [1]. Selection of patients for organ transplantation is based on a number of criteria. Major histocompatability (HMC) genes referred to as human leukocyte antigen (HLA) play a central role in immune response including graft rejection [2]. Selection of the best available ABO-compatible HLA matched organs is one of the most important principles that the organ transplant therapy is organized around. Successful kidney transplantation is the best treatment for patients with end stage renal disease and graft survival is one of the most important measures of success in kidney transplantation. Therefore, to prevent allograft rejection after transplantation, the recipient's immune system has to be suppressed [3].

Rejection

Acute renal allograft rejection is defined as an immunologic process resulting in a detoriation in allograft function that is associated with specific pathologic changes to protect the body from foreign substances and ultimately destroy them [1]. It is now known that CD4 T cells play a central role in the immune response to allograft rejection, and drugs that prevent T cell activation or effector function are therefore potential immunosuppressants which can be used in transplant patients [4]. Immunosuppressants are given to prevent rejection. Most combinations of immunosuppressant during the maintenance phase include either cyclosporine A (CsA) or tacrolimus (Tac), and azathioprine (AZA) or mycophenolate mofetil (MMF) in addition to steroids.

The rejection frequency has decreased over the last years, partly because of the development of more effective medicines. However, there is still high mortality in renal transplant recipient because of cardiovascular diseases; hence it is important to minimize cardiovascular risk factors, such as dyslipidaemia, to further improve both patient and allograft survival.

1.1.2 Hyperlipidaemia treatment and renal transplantation

Dyslipidaemia is much more frequent in solid organ transplant recipients than in the general population [5]. Development of hyperlipidaemia after renal transplantation is related to age, sex, renal function, proteinuria, body mass index, diabetes mellitus, pretransplantation hyperlipidaemia and immunosuppressive therapy like calcineurin inhibitors (CNI), especially CsA which is closely associated with dislipidaemiac adverse effects [5-9].

Hyperlipidaemia has been linked with an increased risk to develop coronary heart diseases in both general population and transplant recipients, and retrospective analyses also suggest a correlation with graft survival [10, 11]. Holdaas *et al*, in a prospective intervention study with fluvastatin showed reduced cardiac mortality and morbidity, but no relation to graft survival [12].

There is a wide agreement that hypercholesterolemia should be treated in renal transplant patient because of their high cardiovascular risk profile and the repeated coexistence of other cardiovascular risk factors such as renal insufficiently, arterial hypertension and diabetes [13]. As renal transplant patients have a very high risk of cardiovascular complication, target low density lipoprotein cholesterol (LDL-C) levels should be less than 100 mg/dL (2.5 mmol/L), similar to the target defined by the National Cholesterol Education Program (NCEP) for high risk population [13]. More than half of all kidney transplant recipients are currently treated with statins and the number is increasing [14]. Wissing *et al* showed that in hypercholesterolemic renal transplant patients treated with CsA, atorvastatin therapy caused a highly significant 30% reduction in total cholesterol and 44% reduction in LDL-C as well as oxidized LDL [15]. These data suggests lipid-lowering therapy with a statin as a rational first step in managing renal transplant patients with hypercholesterolemi [12, 15].

Patients with an organ transplant are multi-medicated and hence are at an increased risk for the potential drug interactions. Particularly the combination of statins and CsA has been shown to increase the risk of muscle toxicity and rhabdomyolysis since the systemic exposure to the statins and their metabolites is increased several fold compared to the general population [16-23], probably partly due to an interaction based on inhibitory affect of CsA on uptake transporters.

1.2 Transporters as a determinant of drug uptake and metabolism

The major function of the liver is to clear endogenous and exogenous substances from the blood. The first step in drug elimination in liver is uptake of drug into hepatocytes through transmembrane transporters in the basolateral membrane. Many different transporters are involved in hepatic transcellular influx and efflux of solutes. The substrate specificity and affinity of the uptake carriers expressed at the basolateral membranes of hepatocytes could therefore play an important role for the determination of the main elimination route of a compound. The rate of transporter-mediated uptake and efflux determines also the rate of renal and hepatobiliary elimination. Transporters are thus important as a determinant of many clearance routes in the body. Even when drugs ultimately undergo liver metabolism, their elimination rate is sometimes limited by the hepatic uptake rate mediated by transporters [24].

Recent studies of drug transport in the liver have provided detailed information on drug transporters, including substrate and inhibitor profiles, and potential drug-drug interactions. An increasing number of drug-drug interactions can be attributed to inhibition or induction of individual transporters mainly expressed in intestinal and liver tissues [24-27].

1.2.1 Transporters

Transmembrane transporters could be broadly classified as uptake/influx or efflux transporters.

Efflux transporters (ATP-binding cassette, ABC)

Members of the ABC transporter family are primary active efflux pumps that reduce intracellular drug concentrations and are classified into seven subfamilies [28], among them are P-Glycoprotein (P-gp), multidrug resistance associated protein 2 (MRP2), sister of P-glycoprotein (SPGP) and Breast cancer resistance protein (BCRP). P-gp are present at a high level in the biliary canalicular membrane of the hepatocytes, the kidney, the small intestine and the blood-brain-barrier [29]. In a CaCo-2 cell line model atorvastatin was shown to be a substrate for P-gp [30]. Simvastatin, lovastatin, pravastatin, cerivastatin and pitavastatin are also shown to be substrates of P-gp [31-34]. Tac and CsA are shown to be inhibitors of P-gp [35-37]. It is also shown that many drugs with affinity to cytochrom P450 (CYP) 3A4 have affinity to this transporter as well [38]. MRP2 express, while highest on the apical domain of the hepatocytes in the liver, but also in the kidney and intestinal entrocytes [39-43]. Chen *et al.* showed that atorvastatin inhibit MRP2-mediated efflux of calcein-AM *in vitro*, which might indicate that atorvastatin is a substrate for this transporter as well [44]. Another inhibitor of MRP2 is shown to be CsA [45, 46]. SPGP, another member of ABC transporter family, is shown to be localized on the canalicular membrane of hepatocytes [47]. Some data indicates that HMG-CoA reductase inhibitors may be substrate of this transporter [48].

Uptake/influx transporters (solute carrier, SLC)

The SLC transporter family consists of 43 gene subfamilies and a total of approximately 300 family members, including ion-coupled transporters, facilitated transporters and exchangers [28, 49]. Subfamilies of the SLC transporter family are Organic Cation Transporter Family (OCT), Organic Anion Transporter Family (OAT) and Organic Anion Transporting Polypeptide Family (OATP).

Organic Anion Transporting Polypeptide Family

Organic Anion Transporting Polypeptides (OATP) are widely expressed in the organs important in drug absorption and distribution including the liver, kidney, intestine, brain and placenta [50]. OATPs are encoded by the gene family SLCO (SLC21) [51], and are Na^+-independent transporters that transport a wide variety of endogenous compounds like bile acids, steroid conjugates, cardiac glycosides, bromosulphophthalien, mycotoxin, leukotriene C_4 and thyroid hormone [52-54]. Drugs like digoxin, methotrexate and statins are also shown to be substrate for OATPs [52, 53]. Clearly the OATP transporter class plays a critical role in hepatic organic anion uptake mechanisms.

Eleven human OATP genes have been described [51] and they have broad and partly overlapping substrate specificity [49]. OATP1A2 (OATP-A) is multispecific and is capable of transporting diverse compounds including bile acid, steroid sulphates, thyroid hormones and opioid peptides [55]. Another OATP member with the potential to alter drug disposition *in vivo* is OATP2B1 (OATP-B), its mRNA has been detected in a number of tissue including liver, lung, kidney, placenta, heart and small intestine [50]. Within the liver, OATP2B1 protein is localized to the basolateral membrane of hepatocytes [56]. Substrate specificity of OATP2B1 appears to be more limited when compared to OATP1B1 or 1B3 (see below). It is possible that OATP2B1 may be an important mediator not just in intestinal or hepatic uptake of a compound but also in facilitating tissue distribution since it is expressed in a number of other tissues than liver and intestine. OATP3A1 (OATP-D) has been identified and characterized mainly as a transporter with a very broad tissue expression profile [57]. Another OATP member OATP1B3 (OATP8), is similar to OATP1B1 (see below) with respect to amino acid composition (80% amino acid identity) and liver specific tissue distribution [58]. In addition, OATP1B3 exhibits substrate overlap with OATP1B1 for compounds albeit with some differences in affinity [56]. OATP1B3 has been demonstrated to be expressed in various gastrointestinal carcinomas and also to play pivotal roles in the uptake of a wide variety of both endogenous and exogenous anionic compounds, including bile acids, conjugated steroids and hormones, into hepatocytes in the human liver [59].

Human liver-specific organic anion transporter 1B1 (OATP1B1, OATP-C, LST-1 or OATP2) is exclusively expressed on the sinusoidal membrane of the hepatocytes in the liver in contrast to all other known transporter subtypes which are found in both hepatic and nonhepatic tissue [50, 52, 60, 61] and was therefore previously called Liver Specific

Transporter 1 (LST-1) [62]. The transport mechanism seems to be anion exchange, but the driving force for OATP1B1 is unknown, though bicarbonate and glutathione have been suggested as exchange mechanisms [63-65]. OATP1B1 has been shown to play an important roll in hepatic uptake and clearance of many albumin-bound amphipathic and organic anion compounds. Endogenous compounds like bile salts, bilirubin and its glucuronides, thyroxin (T_4), triiodothyronine (T_3), bromosulphaophthalien, estradiol-17β-glucuronide, leukotriene C_4 (LTC_4), prostaglandin E_2 (PGE_2) and dehydroepiandrosterone sulphate (DHEAS) are shown to be substrates of OATP1B1 [50, 52, 56].

Drug substrates for OATP1B1 are for instance digoxin [66] and rifampin [67]. It is also involved in the hepatic uptake of statins, including pravastatin [52, 68], cerivastatin [69], and rosuvastatin [70]. Atorvastatin, simvastatin, and lovastatin are effective inhibitors of pravastatin [52], and rosuvastatin [70] uptake by OATP1B1; they are also likely to be substrates for this transporter.

Table 1-1: Human Hepatic OATP involved in statins uptake transport (adapt from Poirier, 2007; [71])

	Human hepatic OATPs			
Statins	OATP1B1	OATP1B3	OATP1A2	OATP2B1
Simvastatin	√	*ND*	*ND*	*ND*
Lovastatin	*Inhib.*	*ND*	*ND*	*ND*
Atorvastatin	√	*ND*	*ND*	*ND*
Fluvastatin	√	√	*ND*	√
Cerivastatin	√	*ND*	*ND*	*ND*
Pitavastatin	√	√	*ND*	√
Pravastatin	√	√	*ND*	√
Rosuvastatin	√	√	√	√

√ data in publication, ND: no data available, Inhib.: inhibits OATP1B1-mediated transport of a model substrate

1.3 Calcineurin inhibitors

Immunosuppressive drugs are used to dampen the immune response in organ transplantation and autoimmune diseases [3, 72]. The essential basic standard and the most effective immunosuppressive drugs in the current immunosuppressive regiments are the calcineurin inhibitors, cyclosporine A (CsA) and tacrolimus (Tac), which target intracellular signaling pathways induced as a consequence of T-cell-receptor activation [73]. Although they are structurally unrelated (Figure 1-1 and 1-2) and bind to distinct, albeit related molecular targets, they inhibit normal T-cell signal transduction essentially by the same mechanism. These drugs bind to an immunophilin (cyclophilin for CsA or FKBP-12 for Tac), resulting in subsequent interaction with calcineurin to block its phosphatase activity. Calcineurin-catalyzed dephosphorylation is required for movement of a component of the nuclear factor of activated T lymphocytes (NFAT) into the nucleus. NFAT, in turn, is required to induce number of cytokine genes, including that for interleukin-2 (IL-2), a prototypic T-cell growth and differentiation factor.

Pharmacokinetic properties of calcineurin inhibitors

CsA and Tac have similar physiochemical properties and elimination pathway. Both undergo extensive intestinal and hepatic metabolism, primary by CYP3A4 and endoluminal transport by P-gp [37, 74-78]. CsA has a greater primary volume of distribution and clearance rate, but no significant difference in bioavailability, absorption rate, and elimination rate as compared to Tac [79]. In clinical practice, Tac is used in doses up to 50-fold lower than those of CsA, mainly duo to its greater potency. The therapeutic concentration of CsA range between 0.1 μmol/L to 1.5 μmol/L, while Tac has a therapeutic range of 5 to 25 nmol/L. However, pharmacokinetic behavior for both drugs is variable between and within individual, and conventional clinical dosing remains unpredictable [79].

1.3.2 Cyclosporine A

CsA, a lipophilic cyclic endocapeptide consisting of 11 amino acids, is produced by the fungus species *Beauveria nivea*. It inhibits the activation of the calcium/calmodulin-activated phosphatase calcineurin [80]. CsA suppresses some humoral immunity, but it is more effective against T-cell-dependent immune mechanisms such as those underlying transplant rejection[81].

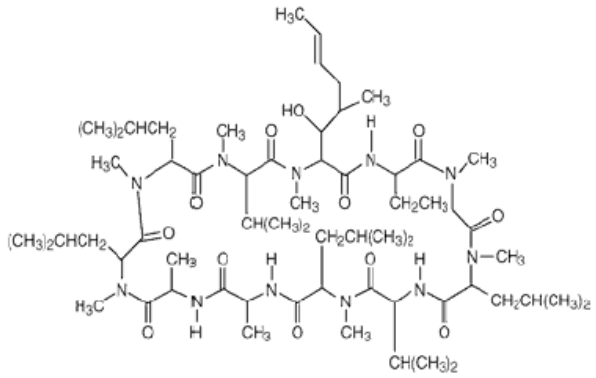


Figure 1-1: Structure of cyclosporine A

Therapeutic uses

Clinical indications for CsA are kidney, liver, heart and other organ transplantation; there are also autoimmune diseases like rheumatoid arthritis and psoriasis [82]. CsA is recognized as the agent that leads to increasing the rate of early engraftment and extending kidney graft survival.

Side effects

The principal adverse reactions to CsA therapy are acute or chronic nephrotoxicity, hypertension, neurotoxicity and hypercholesterolemia [83, 84]. CsA, as opposed to Tac, is more likely to produce elevation in LDL cholesterol [85-87]. Approximately 70% of CsA-treated renal transplant patients have cholesterol serum levels higher than 200 mg/dL (5.18 mmol/L) and 30% higher than 250 mg/dL (6.47 mmol/L) [11]. Additionally, these patients often have an altered lipoprotein profile with an increased fraction of small dens LDL [88].

Drug interaction

CsA interacts with a wide variety of commonly used drugs and it demands close attention. CsA is a well-known substrate of CYP3A4 and P-gp and inhibitor of CYP3A4, OATP1B1, MRP2 and P-gp [35-37, 69, 89-92]. CsA has been reported to be among the compounds which interfere with the hepatic and intestinal uptake of some drugs via OATP1B1, P-gp and MRP2 [30, 52, 69, 93]. The mechanism by which CsA inhibits these transporters is unknown. Any drug that affects microsomal enzymes, especially the CYP3A4 system, may impact CsA blood concentrations [82].

1.3.3 Tacrolimus

Tac is a macrolide antibiotic produced by *Streptomyces tsukubaensis* [94, 95]. Tac has much greater potency than CsA. While Tac is 30-100 times more potent than CsA *in vitro*, maximal inhibition of calcineurin phosphatase *in vivo* has been shown to be greater with CsA than with Tac [96-101].

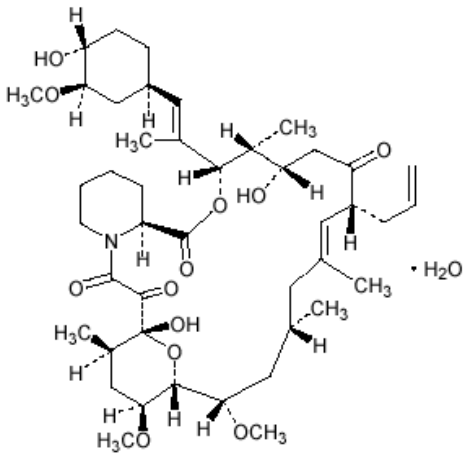


Figure: 1-2: Structure of tacrolimus

Therapeutic uses

Tac is indicated for the prophylaxis of solid organ allograft rejection in a manner similar to CsA and as rescue therapy in patients with rejection episodes despite therapeutic level of CsA [102, 103].

Side effects

Nephrotoxicity, neurotoxicity (tremor, headache, motor disturbance), hyperglycemia and diabetes are all associated with Tac use. Notably, Tac dose not adversely affect the plasma LDL-C level [3].

Drug interactions

Since Tac is metabolized mainly by CYP3A4 and interferer with the uptake and efflux transporters almost in the same way as CsA, the potential interactions described above for CsA also apply, at least theoretically, for Tac [104]. However, not all drug-drug interactions will be of clinically importance since Tac is administrated in a lower concentration and have a higher equilibrium constant (K_i) value on some transporters *in vivo,* compared with CsA.

1.4 Statins

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are a well-established class of drugs in the treatment of hypercholesterolemia. High plasma LDL-C is a risk factor of cardiovascular diseases [105, 106]. Statins are recommended as first-line therapy for hypercholesterolaemia [107, 108], since they have been shown to reduce the risk of cardiovascular morbidity and mortality in patient with or at risk of coronary heart disease in several clinical trails [12, 109, 110].

Mechanism of action

HMG-CoA reductase inhibitors inhibit the synthesis of mevalonate, a rate-limiting step in cholesterol biosynthesis, leading to a reduction in the plasma LDL-C level [111].

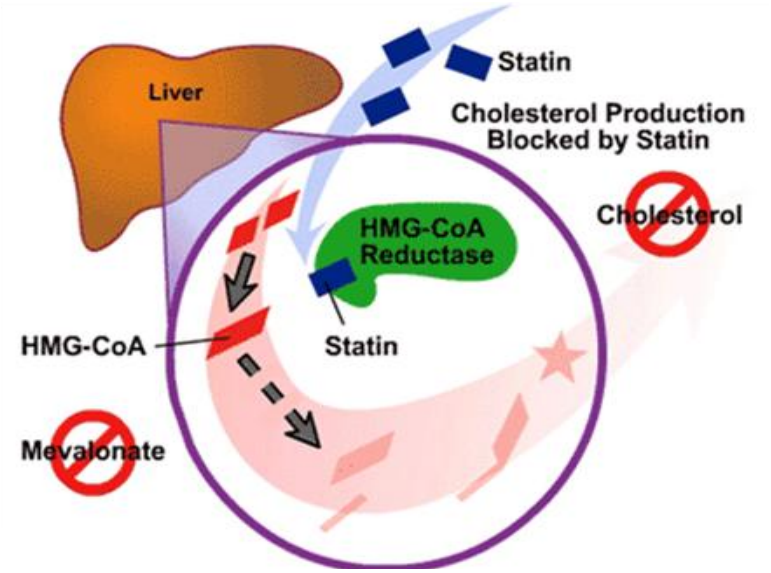


Figure 1-3: The mechanism governing the lipid lowering effect of statins (http://www.medscape.com)

Tolerability and adverse effects

Statins monotherapy is generally well tolerated with a low frequency of adverse events. The common adverse effects associated with statins therapy are relatively mild and often transient, like gastrointestinal symptoms (nausea and abdominal discomfort), headache and rash. The most important adverse effect associated with statins is myopathy which occurs infrequently [3].

The term myopathy designated any noninherited disorder of skeletal muscle that causes proximal muscle weakness. In statin clinical trails, the reported incidence of myopathy is as low as 0.1 to 0.2% [112, 113]. The symptoms may progress toward rhabdomyolysis as long as patients continue to take the drug. Rhabdomyolysis is a syndrome that results from severe skeletal muscle injury and lysis, causing the widespread release of myoglobin with dark brown urine secondary to myoglobinuria [3, 112]. The detailed mechanisms by which statins cause myopathy is not completely understood but some hypothesis have suggested that inhibition of HMG-CoA reductase may directly cause this myotoxicity [114]. The clinical association appears to be dose dependent, and the risk is known to increase when statins are

prescribed in combination with agents that increase the serum concentration of the statins [115, 116].

Biliary excretion of statins

More recently, Matsushima *et al*. clarified multidrug resistance 1 (MDR1) and BCRP as well as MRP2 are involved in the biliary excretion of some statins like pravastatin and cerivastatin [34]. For pravastatin, MRP2 has the most potent transport activity among these efflux transporters while, for cerivastatin, MDR1 and MDR2 have relatively higher transport activity in their experimental systems [34]. Hirano *et al*. reported that pitavastatin is also a substrate of human BCRP, MDR1 and MRP2 [117]. This reports that biliary excretion of statins is mediated by multiple transporters.

Drug interaction

Statins are very selective inhibitors of HMG-CoA reductase and do not show any relevant affinity toward other enzymes [115]. Therefore statins are not disposed to interfere with other drugs at the pharmacodynamic level. However, at the pharmacokinetic level, the available statins have important differences which can give rise to potential drug interaction. The pharmacokinetic differences include half-life, systemic exposure, maximum plasma concentration, bioavailability, lipophilicity, metabolism, presence of active metabolites, and excretion routes [115, 118].

With the exception of pravastatin, which is transformed enzymatically in the liver cytosol, all statins undergo extensive metabolism by the CYP450 isoenzyme systems [119]. Metabolism by CYP450 can affect the potential for drug interaction with statins [115] which can result in markedly increased or decreased plasma concentrations of some drugs within this class. Concomitant use of certain drugs such as CsA, which can interfere with hepatic uptake and biliary excretion of statins as well as CYP metabolism, and increase blood levels of statins and consequently the risk for myopathy [120]. The relationship between altered plasma concentrations and adverse effects like myopathy or toxicity is non linear in some cases [16, 121].

1.4.2 HMG-CoA reductase inhibitors and OATP1B1

The specific transporting systems for the hepatic uptake of many drugs including statins have been characterized. Especially in the case of statins, a transporter mediated drug-drug interaction in the process of hepatic uptake may affect their pharmacological effects because the target organ of statins is the liver. From the pharmacokinetic point of view, the change in the hepatic uptake clearance always directly affects the overall hepatic clearance for this type of drug [24]. To date, it has been reported that pravastatin, cerivastatin, pitavastatin, rosuvastatin, simvastatin, and atorvastatin are substrates of human OATP1B1 [44, 52, 68, 69, 89, 122-125].

1.4.3 Atorvastatin

Atorvastatin, which belongs to the second generation of statins, is a synthetic reversible inhibitor of HMG-CoA reductase. The dosage range used clinically is 10-80 mg/day.

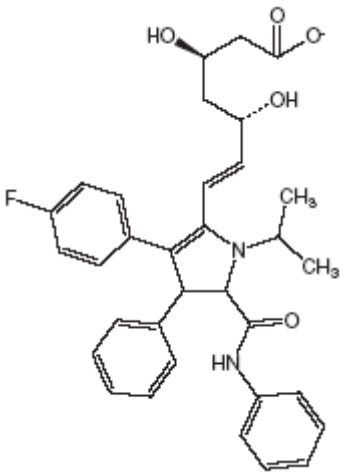


Figure 1-4: structure of atorvastatin

Atorvastatin, with a molecular weight of 546 g/mol and pKa of 4.46, is given orally as the calcium salt of the active hydroxy acid and not as the lactone prodrug [126], but *in vivo* it is in equilibrium with its lactone form [127, 128]. The acid form is a surface active molecular, and it is believed to be important for physiochemical properties, as it will affect its partition into biological membranes and diffusion across the membrane [129].

Metabolism and elimination

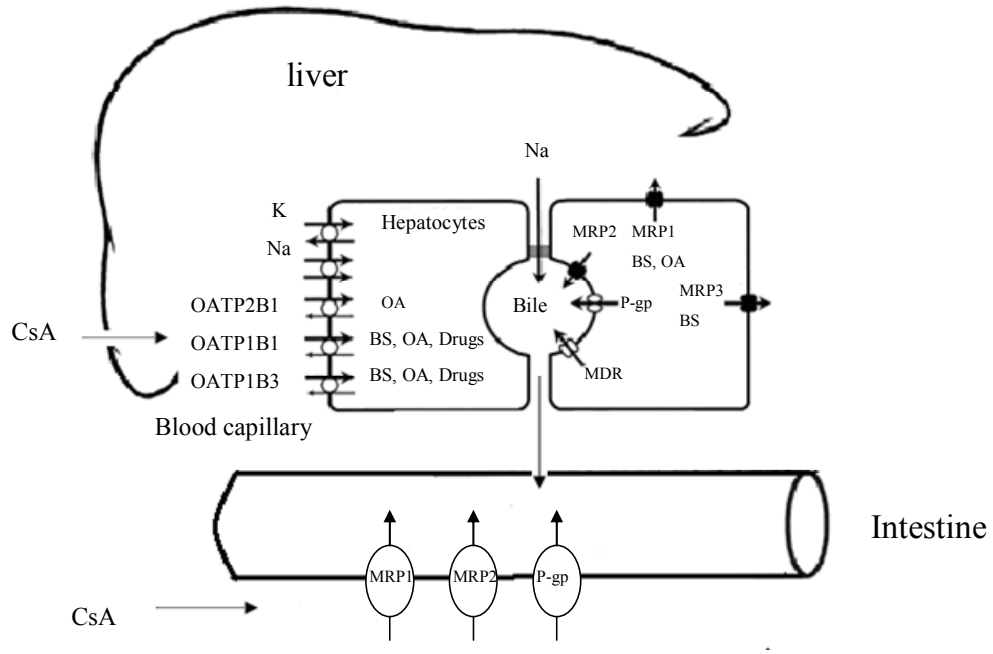
The liver biotransforms all statins including atorvastatin which accounts for their overall low systemic bioavailability. The metabolic pathway of atorvastatin and its metabolites are complex and involve several different steps such as CYP3A4 mediated oxidation, β-oxidation, lactonisation, hydrolysis (chemical and enzymatic) and UGT-mediated glucuronidation. *In vivo* atorvastatin is metabolized by CYP3A4 to two active metabolites, 2-hydroxy-atorvastatin acid and 4-hydroxy-atorvastatin acid, both of which are in equilibrium with their inactive lactone forms [127, 130].

The major route of elimination for atorvastatin and its metabolites is via the biliary route [131]. The renal route is a minor route for elimination in human, as only about 1% of orally administered dose [132]. The apparent total body clearance ($CL_{tot)}$ of atorvastatin is high, about 625mL/min, which corresponds to an E_H of 0.4. This classifies atorvastatin acid as a drug with an intermediate liver extraction [133]. Atorvastatin's clearance is affected not only by the metabolic rate but by the uptake rate as well. Therefore this statin may be susceptible to the OATP1B1-mediated drug-drug interaction [134].

1.5 Combination of statins and immunosuppressive drugs

Among OATP1B1 inhibitors, there are only a few drugs which may cause a drug-drug interaction with coadministrated drugs in clinical situation because of the lower therapeutic concentrations compared with K_i [135]. Among them, CsA is one of the drugs which may affect the pharmacokinetics of other drugs by the inhibition of OATP1B1-mediated hepatic uptake. Most statins (lovastatin, simvastatin, atorvastatin and pravastatin) are metabolised by CYP3A4, and CsA inhibition of CYP3A4 may contribute to the interaction reported between CsA and these statins [16-19]. However, metabolic transformation plays a minor role in some statins like rosuvastatins clearance (CYP2C9 is the principal CYP isozyme involved in the limited metabolism of rosuvastatin), and thus the potential for clinically relevant metabolically mediated drug-drug interaction is low [136, 137]. As previously mentioned, statins are shown to be substrate for OATP1B1 transporters and CsA is known as a potent inhibitor of this transport system in the liver. These theories may explain, at least in part, the drug-drug interaction between CsA and all statins, both those that are metabolised by

CYP3A4 and fluvastatin, cerivastatin and rosuvastatin which are not a substrate for CYP3A4 metabolism [69, 136].



BS: Bile salt

CsA: Cyclosporine A

OA: Organic anion

Figure 1-5: Transporters involved in hepatic and intestinal influx and efflux of solutes.

1.5.1 Pharmacokinetic interaction between atorvastatin and CsA

The combination therapy of atorvastatin and CsA is widely used in the clinical situation. The mechanism of interaction between atorvastatin and CsA is complex, since both drugs are metabolized by CYP3A4 and since CsA has the potential to inhibit several drug transporters expected to be involved in the disposition of atorvastatin [5, 30, 52, 69]. The mechanisms by which CsA might increase the systemic plasma concentration of atorvastatin include enhanced absorption from the gastrointestinal tract (P-gp), reduced hepatic uptake (OATP1B1) and extraction (P-gp), reduced systemic clearance (CYP3A4), or some combination of these effects. Several studies have been shown that CsA has the potential to inhibit hepatic uptake (OATP1B1) and biliary efflux (P-gp) of atorvastatin [30, 52, 69]. On the other hand inhibition of the uptake transporters, which in this case for atorvastatin is OATP1B1, will impair the uptake of atorvastatin into the hepatocytes. Liver hepatocytes are

the main site of distribution for atorvastatin as well as the target organ for its lipid-lowering action. Åsberg *et al*. showed that patients treated with atorvastatin and CsA achieved six-fold higher plasma HMG-CoA reductase inhibitory activity compared to the control group of non-transplant patient receiving only atorvastatin without any significant differences in the lipid lowering effect between the patient and the control group [17]. Lemahieu *et al.* have also shown in their study a 15-fold increase in the systemic exposure to the active acid compound of atorvastatin when CsA was administrated concomitantly [138]. They conclude that the inhibition of OATP1B1 by CsA is responsible for interaction between CsA and atorvastatin. In this study the lack of pharmacodynamic effects despite the increased systemic exposure suggests that CsA not only affects P-gp mediated drug efflux out of cells, but also its specific transport into the hepatocytes, where it exerts its cholesterol-lowering effects.

In the work of Hermann *et al.* an eight-fold higher plasma concentration of atorvastatin was observed in renal transplant patients receiving the combination of atorvastatin and CsA compared to healthy volunteers receiving atorvastatin alone, without any significant difference in terminal half-life ($t_{1/2}$) [139]. They conclude that the increased systemic exposure of atorvastatin is a result of decreased clearance by inhibition of uptake to the liver and biliary efflux since a corresponding decrease in the volume of distribution has been present in order to keep the $t_{1/2}$ unchanged. The liver is likely to be a major contributor to atorvastatin distribution volume. Hence, inhibition of hepatic uptake of atorvastatin will cause a decrease in both distribution volume and clearance.

1.5.2 Interaction between statins (atorvastatin) and CsA versus Tac

In contrast to CsA, only few controlled drug interaction studies have been carried out with Tac. Lemahieu *et al.* performed a pharmacokinetic study of atorvastatin and its metabolites in healthy volunteers treated with CsA and Tac [138]. They indicate that co-administration of CsA significantly increase systemic exposure to atorvastatin acid and its metabolites, while Tac did not have any influence on atorvastatin pharmacokinetics. They conclude that co-administration of Tac and atorvastatin, in general, not result in clinical relevant pharmacokinetic interaction. This is in accordance with other reports like the study of Fehrenbach's group. Fehrenbach has demonstrated in his work that CsA is a competitive inhibitor for OATP1B1-mediated phalloidin transport in stably OATP1B1 transfected

HEK293 cells with a K_i value of approximately 0.5 μmol/L [62]. He shows also that other neutral cyclic compounds like Tac are able to inhibit the OATP1B1-mediated phalloidin transport in the same cell model, but with a K_i value of 3.7 μmol/L. This data indicates that CsA is a more potent competitive inhibitor of OATP1B1 mediated uptake than Tac.

Hirano *et al*. have confirmed the major contribution of OATP1B1 to the hepatic uptake of pitavastatin in human hepatocytes [89]. They have also determined, focusing on OATP1B1, inhibitory effects of various drugs, such as CsA and Tac, on pitavastatin uptake by OATP1B1-expressing cells. Clinical relevance of these drug-drug interactions is discussed by considering R values. (R-value = ratio of the uptake clearance in the absence of inhibitor to that in its presence). R value of CsA is higher than 2.5 (3.55), suggesting that this drug can interact with pitavastatin in a clinical situation in contrast to Tac which has a R value of 1.2.

1.6 HYPOTHESES

Renal transplant recipients treated with the combination of CsA and atorvastatin have been shown to achieve several-fold higher atorvastatin plasma concentration. This interaction has been hypothesised to at least partly be due to inhibition of OATP1B1 mediated uptake of atorvastatin by CsA. The hypotheses of this study are that the cellular uptake of atorvastatin predominantly occurs via OATP1B1 and that CsA inhibit the OATP1B1 mediated uptake of atorvastatin. Tac, another calcineurin inhibitor, has higher K_i value on OATP1B1 than CsA and is administrated in lower concentration *in vivo*; therefore an interaction on OATP1B1 transport will not be present when cells are exposed to clinical relevant doses of Tac.

2. MATERIALS AND METHODS

2.1 Culturing of HEK293 cells

All the work with HEK293 cells was done in a LAF bench using aseptic working procedures. HEK293 cells are human embryonic kidney cells established by F.L. Graham [140]. HEK293 cells transfected with OATP1B1 and pcDNA3 mock cells (cells transfected with the pcDNA3 vector) were provided by Dr. Richard B. Kim at Vanderbilt University.

The cells were incubated at 37 °C in a humidified 5 % CO_2 atmosphere. Culturing of the cells was performed in 75 cm^2 flasks and six-well plates in high glucose (4.5 g/L) Dulbecco's modified eagles medium (DMEM). The growth medium (DMEM) was changed every other day. The cells were grown in 10 mL and 2 mL medium per flask and well, respectively. For experiments, culturing was performed on poly-D-lysine coated six-well plates to enhance attachment to the wells. The coating was performed by adding 0.5 mL poly-D-lysine to each well and incubating at room temperature for an hour. The remaining coat medium was then removed and the wells were left to dry at room temperature.

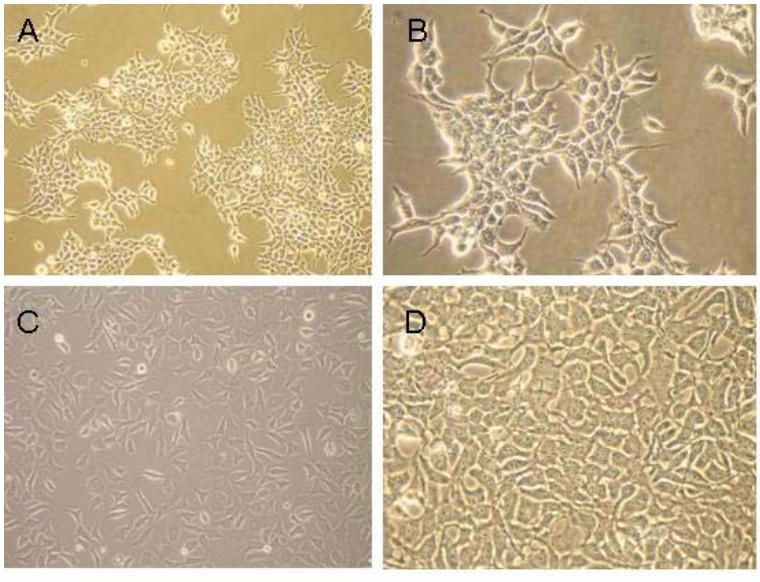


Figure 2-1: Picture of HEK293 cells cultured in 75 cm^2 flasks; OATP1B1 10 x magnification (A), OATP1B1 20 x magnification (B), Mock 10 x magnification (C), and Mock 20 x magnification (D)

2.1.1 Thawing HEK293 cells

An ampoule of 1 mL HEK293 cells, both OATP1B1 and mock, was rapidly thawed at 37 °C. The cell suspension was transferred to a tube containing 5 mL prewarmed growth medium and centrifuged at 160 g (Kubota 2010 centrifuge, Medinor, Oslo, Norway) for 5 minutes. The pellet was resuspended in 5 mL growth medium and transferred to a 75 cm^2 culture flask containing additional 5 mL prewarmed (37 °C) growth medium. Growth medium was changed every other day until approximately 90 % confluence.

2.1.2 Splitting of HEK293 cells

The cells were splitted at 90 % confluence, which was usually every fourth or fifth day. Each flask then contained approximately $5x10^6$ cells. At that point, the old growth medium was removed and the cell-layer were washed with 10 ml Hanks' Balanced Salt Solution (HBSS). Subsequently the cells were detached from the flask by adding 1.5 mL trypsine-EDTA diluted with HBSS (1:1, v/v). After 1-2 minutes incubation at 37 °C, the cells were brought into suspension by tapping the sidewalls of the flask, and 4.5 mL growth medium was added to the cell-suspension in order to deactivate trypsin. The suspension was then transferred to a tube and centrifuged for 5 minutes at 160 g (Kubota 2010 centrifuge, Medinor, Oslo, Norway). The supernatant was removed and the cell pellet was resuspended in 6 mL growth medium. The cells were further seeded into flasks or coated six-well plates, at densities of $2.0x10^6$ and $5.0x10^5$ cells per flask and well, respectively.

2.2 Gene expression analysis by polymerase chain reaction

2.2.1 Methodological principle

Gene expression of OATP1B1 in both transfected and mock cells was performed using reverse transcriptase (RT) PCR analysis. Total RNA was first isolated from respective cells and subsequently reversetranscribed to complementary DNA (cDNA). Thereafter, amplification of the cDNA sequence of interest was performed by use of specific primers and PCR technique. The DNA fragments were separated by agarose gel electrophoresis and visualized by ethidium bromide (EtBr) staining.

2.2.2 Isolation and quantification of total RNA

Isolation of total RNA was performed by use of Agilent Total RNA Isolation Mini Kit®. One day before cell lysis, the cells were stimulated with a final concentration of 5 mM sodium butyrate in order to increase gene expression of OATP1B1.

Splitting of cells was performed as described in section 2.1.2, but this time the cell suspension was centrifuged at 200 g (Kubota 2010 centrifuge, Medinor, Oslo, Norway) for 5 minutes and the supernatant was removed. Further, washing of the cell pellet was performed three times by adding 1 mL 1xPBS and centrifuged at 200 g for 5 minutes between each time. Subsequently, 300 µL of lysis solution (added 10 µL β-mercapoethanol / mL) was added to each tube. The cell homogenate was transferred to mini prefiltration column and centrifuged through the column for 3 minutes at 16 000 g (universal 32R centrifuge, Hettich Zentrifugen, Tuttlingen, Germany). Thereafter, an equal volume (300 µL) of 70 % ethanol was added to the filtrate, mixed carefully and incubated for 5 minutes. The ethanol/lysis mixture was added to the mini-isolation column and centrifuged for 30 seconds at 16 000 g. The flow-through was discarded and the RNA loaded column replaced into the same collection tube. An amount of 500 µL of wash solution was added to the mini-isolation column in the collection tube, then centrifuged for 30 seconds at 16 000 g. The washing process, followed by centrifuging was repeated one more time. The flow-through was discarded and the column was centrifuged for 2 minutes at 16 000 g. Elution of purified RNA was performed by adding 25µL of nuclease-free water. After incubation for at least 1 minute, the column was centrifuged for 1 minute at 16 000 g.

2.2.3 Detection of RNA purity and concentration

The concentration of RNA in the samples was determined by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) by spectrophotometer (Gene Quant Pro DNA/RNA Calculator®, Amersham Pharmacia Biotech). Absorbance measurement at 260 nm wavelength permits the direct calculation of nucleic acid concentration in a sample. The ratio between the absorbance at 260 nm and 280 nm (A_{280}) gives an estimate of the purity of RNA compared to proteins. A pure RNA sample has an A_{260}/A_{280} ratio in the range of 1.9-2.1.

For determination of RNA concentration, 2 µl of the RNA solution was diluted with 68 µL nuclease-free water (dilution factor 1:35).

The total RNA concentrations were calculated from the formula:

OD_{260} x 40 x 35 (dilution) /1000 = concentration of total RNA (μg/μL)

The volume needed to give 1 μg RNA was calculated and used for RT cDNA synthesis.

2.2.4 Reverse Transcriptase (RT)

In order to be able to apply PCR analysis to the study of mRNA, mRNA is transcribed into the much more stable cDNA. cDNA is synthesized by the enzyme SuperScript III reverse transcriptase which is an RNA-dependent DNA polymerase. The 3' end of the mRNA acts as a start point for the polymerase, and a short double-stranded sequence is needed. In a mixture of nucleotides (dNTP mix), primers (oligo(dT)), template total RNA and the appropriate salt, temperature and pH conditions, the reverse transcriptase will synthesize a complementary DNA on the mRNA template.

The making of complementary DNA (cDNA) from RNA was done by means of the TagMan® Reverse transcription reagents kit (Applied Biosystems). Reverse transcription was run on GeneAmp PCR systems 9700 (Applied Biosystems, Foster city, California, USA). 1 μg of total RNA was added to each of the reaction tubes containing DEPC-H_2O and TagMan® reaction mix (appendix, section 7.5.2), giving a total volume of 20 μL.

Three steps comprise the reaction:

- Annealing of primers to RNA: 25 °C for 10 minutes
- cDNA synthesis: 37 °C for 60 minutes
- Inactivation of enzymes: 99 °C for 5 minutes

The synthesized cDNA was stored at – 20 °C.

2.2.5 Polymerase Chain Reaction (PCR)

The newly formed cDNA must be amplified in order to reach detectable levels. This is accomplished with PCR, which is a powerful and sensitive method of enzymatically amplifying a selected DNA sequence *in vitro*. The method is based on the use of DNA

polymerase to copy a DNA template in repeated cycles, giving an exponential increase in DNA-product. The polymerase is guided to the sequence to be copied by short synthetically produced oligonucleotide primers that hybridize to the DNA template at both extremities of the desired DNA sequence. These primers induce replication of each strand of the original DNA. The PCR process is carried out in automated thermal cycles, which heat and cool the reaction tubes to the precise temperature required. The double-stranded DNA is first heated-denatured at high temperature, then annealed at a lower temperature to two oligonucleotide primers, and finally extended at intermediated temperature.

PCR was performed by use of AmpliTaqGold® with GeneAmp® reagents. PCR was run by use of GeneAmp PCR System 9700 (PE Applied Biosystems, Foster city, California,USA).

10 µL cDNA sample (RT-product) was added to each of the reaction tubes containing GeneAmp® reaction mix (appendix, section 7.5.2), giving a total volume of 50 µL.

The amplification program was initially started at 94 °C for 3 minutes, thereafter 30cycles of the following three steps:

- Denaturation: 94 °C for 30 seconds
- Annealing: 50 °C for 20 seconds
- Extension: 72 °C for 20 seconds

} x 30 cycles

And finally 72 °C for 1 minute.

In addition to the OATP1B1 receptor genes, β-actin (housekeeping gene) was run for each sample. β-actin mRNA is expressed at moderately abundant levels in most cell types, and its broad band is used as a control to see if the PCR reaction is successfully accomplished and to adjust for differences in added total RNA in each vial.

Primers:

OATP1B1 has a fragment length of 97 base pairs (bp) with the following primers:

Forward OATP1B1: 5'-GTA CCA CTT TCT TAT TGC AAC TCA GAC T-3'

Reverse OATP1B1: 5'-GAC AGG GTG AGA TGT AAG TTA TTC CA-3'

β-actin has a fragment length of 62 base pairs with the following primers:

Forward β-actin: 5'- ACC GAG CGC GGC TAC A-3'

Reverse β-actin: 5'- TCC TTA ATG TCA CGC ACG ATT T-3'

2.2.6 Agarose gel electrophoresis

Agarose gel electrophoresis is performed to separate and identify the DNA fragments. At neutral pH, DNA is negatively charged and in an electrical field it will migrate toward the positive electrode (anode). The migration rate of DNA in the gel is dependent on the conformation and size of DNA, agarose concentration, buffer concentration and the electric potential applied. To determine the size of separated DNA molecules, the samples are compared to the DNA standard (DNA Ladder) which is run on the same gel. The gel is prestained with the UV-fluorophore ethidium bromid (EtBr) which intercalates between the basepairs of the DNA double helix, and allows the DNA fragments to be visualized under the exposure of UV-light.

DNA fragments obtained in RT-PCR were separated on a 2% agarose (appendix, section 7.5.3) gel by electrophoresis. The warm solution of 2% agarose gel was poured into the gel mould fitted with the appropriated comb and the gel was allowed to set at room temperature. The gel was transferred to an electrophoresis chamber, covered with 1xTBE buffer and the comb was removed. Subsequently, 15 µL of each PCR product was mixed with 3 µL loading dye buffer and applied to the wells. 15 µL of 0.1 µg/µL standard low molecular weight DNA ladder (LMW-Ladder) was applied to the first well. The electrophoresis was run at 100 V (Power Pac 200, Bio-Rad Laboratories Inc.) for approximately 1.5 hours. The DNA was visualized by use of UV-light.

2.3 Protein quantification

The Bio-Rad Protein Assay was used for determining the concentration of total protein in each sample based on the original works of Bradford [141]. This method is based on the colour change of a dye (Coomassie® Brilliant Blue G-250) when it binds to basic and aromatic amino acids in an acidic environment. The absorbance maximum shifts from 465 nm to 595 nm when binding occurs. The absorbance measured at 595 nm is proportional to the amount of dye bound, and thereby also the protein concentration.

A standard curve derived from bovine serum albumin (BSA) was used in the range of 0-75 µg/mL. The standards were diluted in distilled water to known concentrations. The protein samples were sonicated for 15 minutes using a VibraCell™ ultrasonic processor (Vibra cell™, Sonic & Materials Inc, Danbury, Connecticut, USA). Duplicates of 50 µL of each sample, as well as the protein standards were added to a 96-well microtiter plate. Subsequently, 150 µL of the Bio-Rad protein assay dye reagent (diluted 1:2.75 in distilled water) was added to each well and absorbance measured at 595 nm. The protein concentration was calculated by a microplate reader/spectrophotometer Wallac 1420 Victor 3™ Multilable counter (PerkinElmer Life and Analytical Sciences Inc, Boston, Massachusetts, USA).

2.4 Protein analysis by Western blot technique

2.4.1 Methodological principles

Western blotting is a protein analysis technique used to detect specific proteins in a complex protein mixture. The protein samples are denaturated and separated by electrophoresis on a polycrylamide gel. The proteins are then transferred from the gel to a polyvinyllidine fluoride (PVDF) membrane where they are soaked in a solution containing antibodies that recognize and bind specifically to their corresponding antigens.

This analysis involves the following steps:

- Harvesting/lysis of treated cells
- Electrophoresis
- Transferring of proteins from gel to PVDF membrane
- Blocking and protein detection with antibodies
- Imaging of proteins using chemiluminescence

2.4.2 Harvesting/lysis of treated cells

Both OATP1B1 and vector transfected (mock) cells were cultured in 75 cm^2 flasks with 10 mL growth medium. One day before cell lysis, some of the cells were stimulated with 5 mM sodium butyrate. The cells were rinsed twice with HBSS. To detach them from the culturing flasks, 300 µL sample buffer was added. The cells suspended in sample buffer, one parallel, were then transferred to a heat-resistant vial, and cooked on a water bath for 5 minutes for complete lysis and denaturation of the proteins. β-mercaptoethanol (10% v/v) and brom-phenol-blue (5% v/v) was added before the cells was frozen at -20 $^{\circ}C$

2.4.3 Electrophoresis

The purpose of the electrophoresis method is to separate proteins according to the size and charge. The anionic detergent sodium dodecylsulfate (SDS) denatures proteins by conferring a net negative charge proportional to their length, allowing separation as they move across a

polyacrylamide gel network functioning as a sieve to help "catch" the negatively charged molecules as they are transported by the electric current towards the positive end.

The buffer used was Laemmli system, which is a discontinuous buffer system. In a system like that, a non restrictive large pore gel, the stacking gel, is layered on the top of the separating gel. Due to the higher acrylamide percentage in the separating gel, the proteins will be up-concentrated on the interface between the two gels resulting in increased protein resolution.

Two pieces of 10% and 12% separating gel solution were prepared (as described in appendix, section 7.5.7). The solution was pipetted to the glass sandwich to a level about 1.0 cm from the top. The glass sandwich was made of two glass plates and to metal plates making two cassettes, separated by two spacer strips at the edges and champed together to make a tight seal. To prevent formation of meniscus on the top of the gel, 200 µL of isobuthanol was applied to the gel containing cassettes before polymerization. The gels were allowed to polymerize for 45 minutes. Thereafter, isobuthanol was poured from the surface and gels were rinsed with distilled water. Stacking gel (4%) was prepared (according to section 7.5.7), and added to the sandwich. Combs were inserted into each gel. The gels were allowed to set for another 40 minutes. Then, the gel-containing cassettes were rinsed with distilled water and placed in an electrophoresis chamber. The chamber was then filled with Tank buffer until the cassettes were immersed in buffer. The combs were removed from the gels and a volume of 5 µL protein standard and 15 µL of samples were applied to the wells and separated by electrophoresis at 200 V for 1 hour and 20 minutes.

2.4.4 Transferring of proteins from gel to PVDF membrane

To transfer the separated proteins onto a polyvinylidene fluoride (PVDF) membrane, the gels were placed into a transfer cell ("sandwich"). Gels, blotter papers, nitrocellulose membranes and cellophane sheets were all soaked in blotting buffer and then arranged in a "transphor sandwich" (fig 2-2). The polyamide gels were placed face to face with the membrane. Gels were blotted at 100 mA for 45 minutes, with electric field applied perpendicular to the gels, making the proteins move out to the membrane. To verify successful protein transfer after blotting, membranes were stained for 1 minute with Ponceau S-solution and rinsed with distilled water.

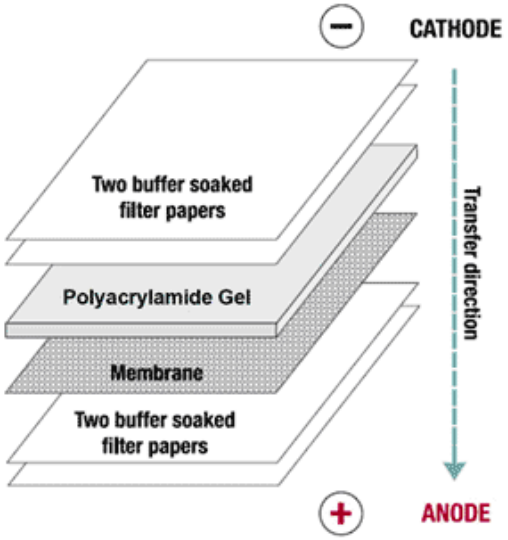


Figure 2 -2: Arrangement of the filter paper, membrane and gel for transphor.

2.4.5 Blocking and protein detection with antibodies

To avoid non-specific binding of antibodies used in later stages, the membranes need to be "blocked". For this purpose membrane were placed in Tris-Tween-Buffered-saline-solution (T-TBS solution) for 10 minutes first. Next 10-15 mL of Blotto-solution was added to each membrane and they were allowed to set for 60 minutes.

In order to visualize the immobilized protein band of interest on the membrane, they first were incubated with a primary antibody specific for the protein (antigen) of interest, followed by incubation with a secondary enzyme-coupled antibody directed towards the primary. The membranes were incubated with 15 mL of primary antibodies (appendix, section 7.4) in a cold room over night. Membranes were then washed for 3x10 minutes in blotto prior to addition of the secondary antibodies, followed by additional 1 hour of incubation at room temperature. Membranes were then washed 3x10 minutes in blotto and 2x10 minutes in T-TBS.

2.4.6 Imaging of proteins using chemiluminescence

ECL™ Western Blotting System uses horseradish peroxidase (HRP) conjugated secondary antibodies for luminal-based detection of Western blots. HRP locally catalyzes the conversion of ECL™ detection reagents into a sensitized reagent, which generates an intense

chemifluorescent signal at 440 nm. This chemifluorescent signals can be detected on a fluorescence imager with the appropriate filters [142].

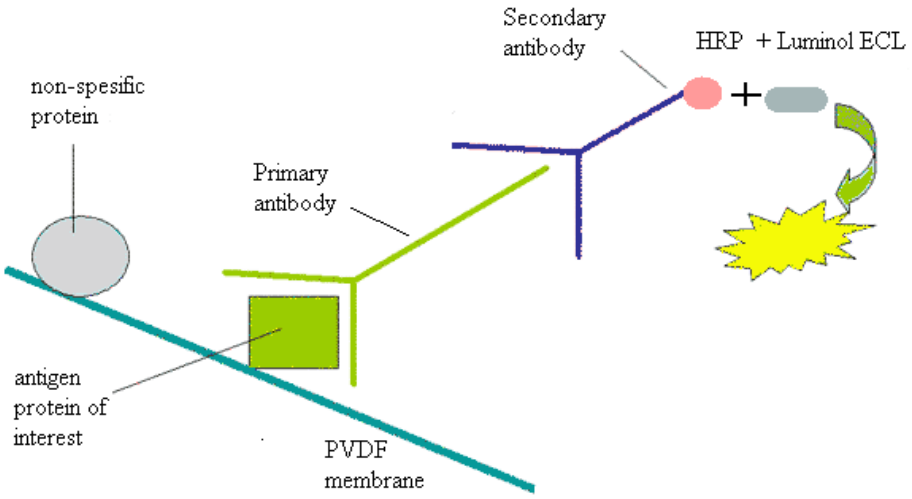


Figure 2-3: The principle of chemiluminescent immunodetection. HRP, horseradish peroxidase

ECL Plus™ detection kit was used. Equal amounts of chemifluorescent reagents (8 mL total volume) were mixed and added to the membranes, which were then packed in plastic folium. The membranes were transferred to a film cassette and exposed to film for different amount of time (Appendix, section 7.4). Then the film was developed and fixated.

2.5 Uptake experiments

The uptake of atorvastatin in OATP1B1 and vector (mock) transfected HEK293 was investigated. The effect of CsA and Tac on this cellular uptake was examined in OATP1B1 transfected and mock HEK293 cells by pre- and co-incubation with CsA and Tac.

HEK293 cells were used in experiments two day after seeding onto pre-coated six-well plates. The cell cultures were incubated with 5 mM sodium butyrate to increase gene expression of OATP1B1, 24 hours before the uptake studies in HEK293 cells.

2.5.1 Studies of cellular uptake of atorvastatin acid

In order to investigate the uptake of atorvastatin in OATP1B1 transfected and mock cells, the cells were incubated with 1 mL medium containing various concentration of atorvastatin acid (range: 0-10 µmol/L, table 2-1) with different incubation time (range: 1-40 minutes, table 2-2) at 37 °C in a humidified 5% CO_2 atmosphere. The experiments were carried out with three parallels at each atorvastatin level. After the incubation time, the medium was removed and the cells were washed twice with 2 mL ice-cold PBS buffer. Thereafter, 500 µL acetonitrile - water (90:10, v/v) was added twice for lysation and protein precipitation. Subsequently, 20 µL internal standard (I.S.), 0.5 µg/mL methaqualone, and 20 µL 0,05 µg/mL deuterated atorvastatin acid and lactone (d_5 atorvastatin) was added to each well. Harvesting of cells was performed, after each addition of acetonitrile - water, by scraping the cells from the bottom of the wells using a plastic cell scraper. The cell lysates were transferred to tubes. The cell suspension were frozen at - 70 °C for at least an hour in order to complete lysation.

The cell suspensions were thawed and centrifuged (Universal, 32R centrifuge, Hettich Zentrifuged, Tuttlingen, Germany) for 10 minutes at 20 000 g at 4 °C. The supernatant was transferred to new vials and evaporated to dryness by use of either nitrogen (N_2) gas at room temperature or Speed Vac. The residue was reconstituted in 120 µL mobile phase A and centrifuged for 10 minutes 20 000 g at 4 °C. The supernatant was then transferred to HPLC vials for injection in the HPLC-MS/MS system.

The protein pellet was reconstituted in 250 µL distilled water for protein quantification.

Table 2-1: concentration curve for OATPB1B1 transfected and mock cells

Concentration of atorvastatin, µmol/L	*Incubation time, minutes*
0	5
0.25	5
0.5	5
0.75	5
1.0	5
1.5	5
2.0	5
3.0	5
5.0	5
10.0	5

Table 2-2: Time curve for OATPB1B1 transfected and mock cells

Incubation time, minutes	*Concentration of atorvastatin, µmol/L*
1	1.5
3	1.5
5	1.5
10	1.5
20	1.5
40	1.5

2.5.2 Studies of the effect of CsA and Tac on cellular uptake of atorvastatin

Effect of CsA on cellular uptake of atorvastatin

To investigate the effect of CsA on cellular uptake of atorvastatin, both mock and OATP1B1 transfected HEK293 cells were incubated with 1 mL medium containing various concentration of CsA (0-50 µmol/L) for an hour at 37 °C in a humidified 5% CO_2 atmosphere. The experiments were carried out with three parallels at each concentration of CsA. Following pre-incubation, the medium was replaced by 1 mL medium containing both 3 µmol/L atorvastatin and CsA (0-50 µmol/L, table 2-3). The cells were then incubated for 5 minutes at 37 °C. The rest of the experiment was performed in the same way as described above for uptake of atorvastatin (section 2.5.1).

Table 2-3: Concentration range of CsA used in CsA inhibition experiments

Concentration of CsA, µmol/L	*Concentration of atorvastatin, µmol/L*	*Incubation time, minutes*
0	3	5
0.25	3	5
0.5	3	5
0.75	3	5
1.0	3	5
1.5	3	5
2.0	3	5
3.0	3	5
5.0	3	5
10.0	3	5
20.0	3	5
50.0	3	5

Effect of Tac on cellular uptake of atorvastatin

To investigate the effect of Tac on cellular uptake of atorvastatin, both mock and OATP1B1 transfected HEK293 cells were incubated with 1 mL medium containing varius concentration of Tac (0-10 µmol/L, table 2-4) at the same condition as for the CsA experiment.

Table 2-4: Concentration range of Tac used in Tac inhibition experiments.

Concentration of Tac, µmol/L	*Concentration of atorvastatin, µmol/L*	*Incibation time, minutes*
0.00	3	5
0.05	3	5
0.10	3	5
0.15	3	5
0.25	3	5
0.35	3	5
0.50	3	5
0.75	3	5
1.00	3	5
2.00	3	5
5.00	3	5
10.00	3	5

2.6 HPLC-MS/MS

2.6.1 Methodological principles

HPLC-MS is high-performance liquid chromatography coupled to mass spectrometry. It is a powerful technique that has very high sensitivity, specificity and detection limits in the pg level. In the MS step the analytes are fragmented and the ions of interest are selected according to their mass-to-charge ratio (m/z). Separation of the analytes by use of HPLC precedes the MS detection to further enhance sensitivity and specificity of the method.

HPLC-MS analysis of intracellular concentration of atorvastatin was performed using a method described and validated by Hermann *et al*. [143]. Since atorvastatin acid undergoes

interconversion to the lactone form also *in vitro,* both the intracellular concentration of atorvastatin acid and lactone was determined. The HPLC-MS/MS equipment consisted of a Agilent Technologies 1200 autosampler, a Agilent Technologies 1200 quarternary pump, and a Agilent Technologies 6310 MS/MS ion trap. The analysis of the chromatograms was performed using the software Chemstation for LC 3D systems, Rev. B.01.03 (204).

A volume of 120 µL sample was placed in the chilled autosampler tray and 100 µL of each sample was injected in the HPLC system. A C_{18} analytical column (Omnisphere C_{18}, 3µm, 30x2mm, Varian, Lake Forest, California, USA) with a chromoguard guard column (ChromoSepGaurd Column SS, 3µm, 10x2mm, Varian, Lake Forest, California, USA) was used for chromatographic separation of the analytes.

The analytes were eluted by a gradient mobile phase system consisting of mobile phase A and mobile phase B (Appendix, section 7.5.6). The total run time per sample was 28 minutes. A linear change from 100% mobile phase A to 60% mobile phase A and 40 % mobile phase B was performed during the first 5 minutes after sample injection. This mobile phase composition was kept for 15 minutes, and then linearly reversed back to 100% mobile phase A within 1 minute. The flow was kept stable at 0.2 mL/min throughout the analysis, except for from 21^{th} to 26^{th} minute which the flow was 0.4mL/min. The column was re-equilibrated with mobile phase A for 7 minutes (from 21^{th} to 28^{th} minute), before a new sample was applied to the system.

Uptake curves of atorvastatin were obtained on the basis of arbitrary units (MS/MS signal of atorvastatin in relation to internal standard).

Table 2-5: Precursor and product ion masses and duration of each segment in the MRM tandem MS detection.

Segment	*1*	*2*	*3*	*4*	*5*
Compound	*Waste*	*I.S. methaqualon*	*Atorvastatin acid + I.S (d_5 acid)*	*Atorvastatin acid + I.S. (d_5 lactone)*	*Waste*
Retention time duration (minutes)	0-2.5	2.5-7	7-16.5	16,5-22.5	22.5-28
Product ion (m/z)	-	132.0	440.2 + 445.5	448.2 + 453.3	-
Precursor ion (m/z)	-	251.5	559.0 + 564.5	541.2 + 546.7	-

3. RESULTS

3.1 OATP1B1 gene expression analysis

Gene expression of OATP1B1 was investigated using RT-PCR analysis. The DNA fragments were separated by agarose gel electrophoresis (Figure 3-1). The OATP1B1 transfected HEK293 cells (lane 3-4) showed expression of OATP1B1 mRNA, in contrast to non-transfected HEK293 cells (lane 1-2), where no expression of OATP1B1 was observed. As expected, the mRNA expression level of OATP1B1 was higher in OATP1B1 transfected HEK293 cells stimulated with sodium butyrate prior to mRNA isolation (lane 4) than in cells not subjected to sodium butyrate stimulation (lane 3).

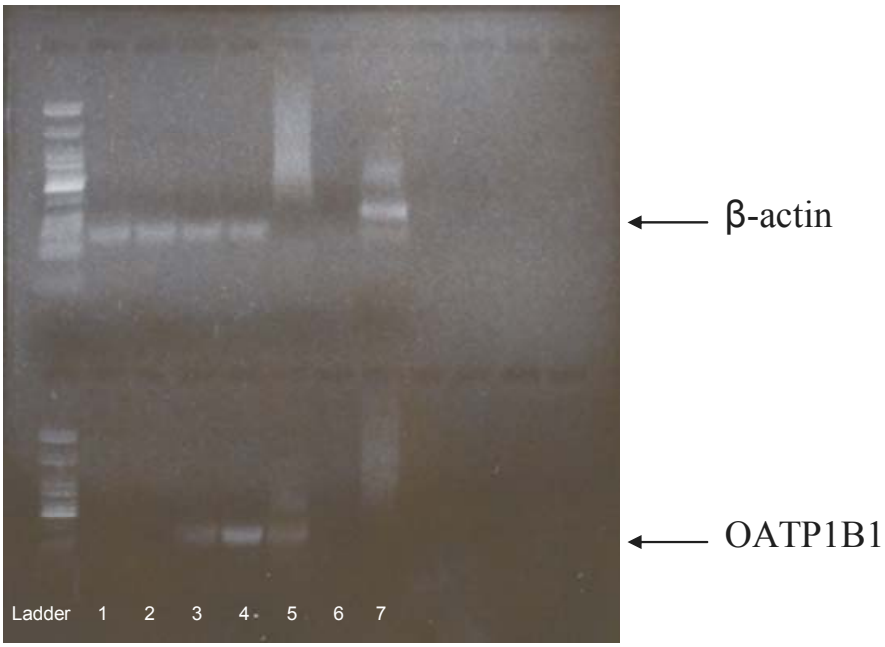


Figure 3-1: RT-PCR products separated on 2% agarose gel. 1: HEK293 mock cells, 2: HEK293 mock cells stimulated with sodium butyrate, 3: OATP1B1 transfected HEK293 cells, 4: OATP1B1 transfected HEK293 cells stimulated with sodium buttyrate, 5: positive control for β-actin and OATP1B1respectively, 6: negative control, 7: positive control for OATP1B1 and β-actin respectively.

3.2 OATP1B1 protein expression analysis

Western blots of OATP1B1 protein expression in both transfected and mock HEK293 cells are shown in figure 3-2. The OATPB1B1 protein has a molecular weight of 90 kDa [144].

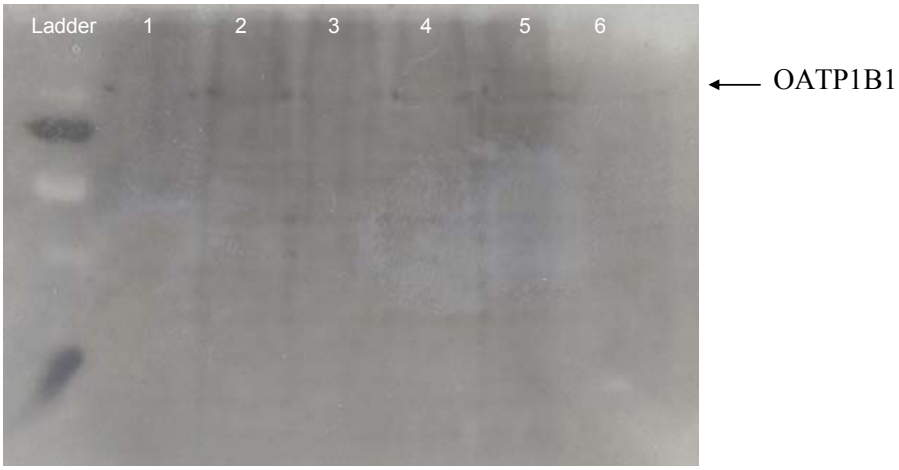


Figure 3-2: Western blot gel of OATP1B1in mock and OATP1B1-overexpressed HEK293 cells (SDS-polyacrykamide gel). Lane 1: HEK293 OATP1B1 transfected cells, 2: HEK293 OATP1B1transfected cells stimulated with sodium butyrate, 3: HEK293 mock cells, 4: HEK293 mock cells stimulated with sodium butyrate, 5: HEK293 OATP1B1 transfected cells stimulated with sodium butyrate and boiled for 5 minutes, 6: HEK293 mock cells stimulated with sodium butyrate and boiled for 5 minutes.

The bands from both OATP1B1 transfected and mock cells showed a slightly higher molecular weight than the expected 90 kDa. As expected, the protein expression level of OATP1B1 was somewhat higher in OATP1B1 transfected HEK293 cells stimulated with sodium butyrate than in HEK293 mock cells (figure 3-2, bands 2 and 4). All bands are of low intensity but a 10% difference in favour of the over expressed cells was shown between the OATP1B1 transfected and mock cells (bands 5 and 6) when analysing them with the GeneTools program from SynGene. However, a lot of unspecific binding to other proteins than OATP1B1 were present.

3.3 Cellular uptake of atorvastatin

Uptake of atorvastatin in both OATP1B1 transfected and mock HEK293 cells were investigated both as a function of incubation time (0-40 minutes) and different concentrations of atorvastatin (0-10 µmol/L). The HPLC-MS/MS chromatograms of the intracellular atorvastatin concentration were integrated with respect to area under curve (AUC) and presented as arbitrary unites (related to AUC of internal standard). A typical HPLC-MS/MS chromatogram is illustrated in figure 3-3.

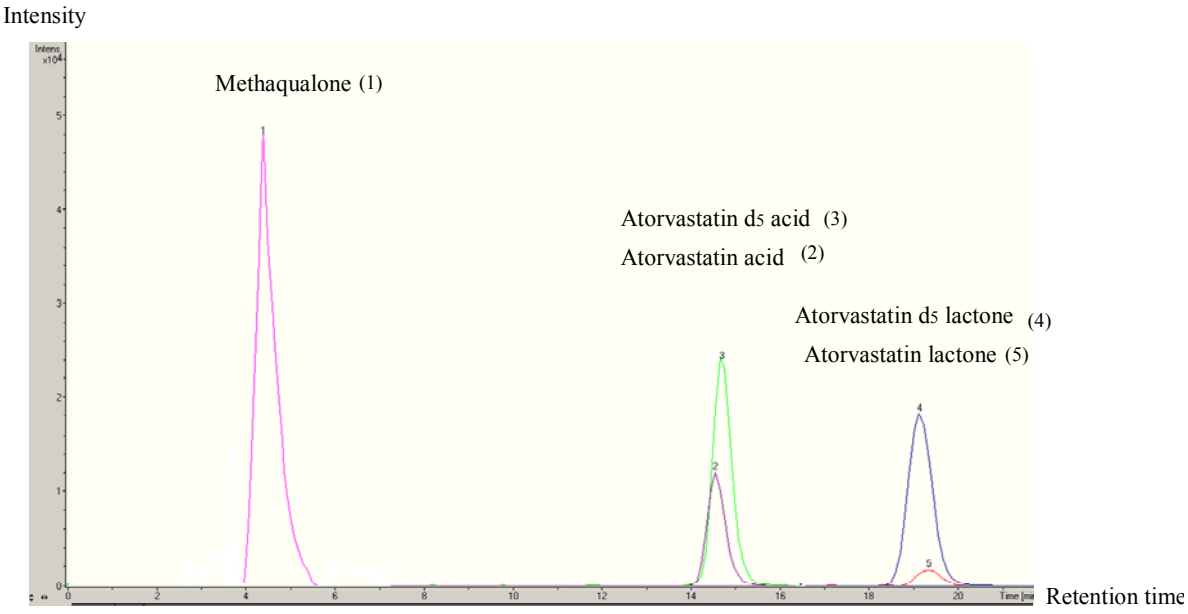


Figure 3-3: Representative HPLC-MS/MS chromatogram. The first peak shows methaqualone (used as an alternative I.S.) with retention time at almost 4.5 minutes, the second and third peak show atorvastatin acid and atorvastatin d_5 acid (used as I.S.) with retention time at almost 14.5 minutes, the fourth and fifth peak show atorvastatin lactone and atorvastatin d_5 lactone with retention time at almost 19 minutes.

The uptake experiments in HEK293 cells indicates an approximately 2.5-fold higher uptake of atorvastatin in OATP1B1 transfected cells than in mock cells as shown in figure 3-4.

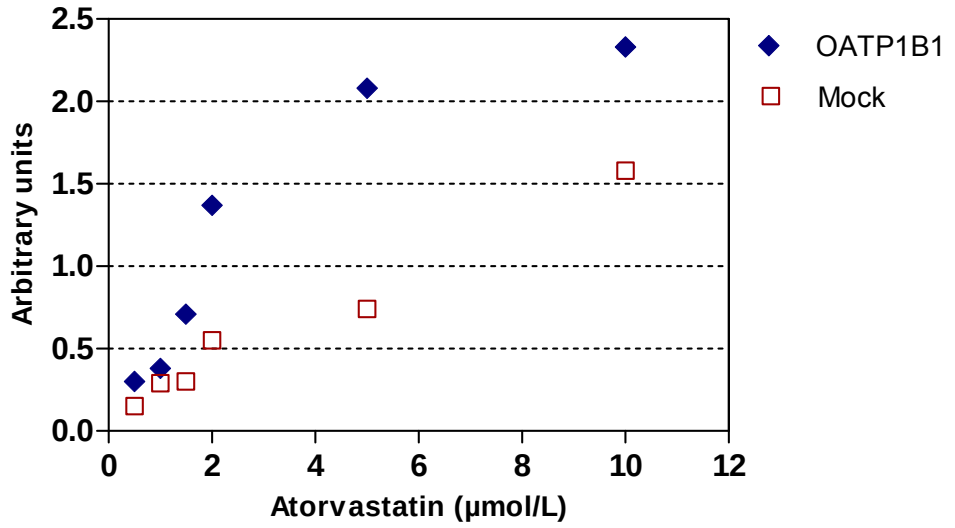


Figure 3-4: Uptake of atorvastatin in OATP1B1 transfected and mock HEK293 cells was examined. Uptake curves for atorvastatin were obtained on the basis of arbitrary units: MS/MS signal of atorvastatin in relation to MS/MS signal of internal standard.

The differences in cellular uptake of atorvastatin observed between OATP1B1 transfected and mock cells is noticeable greater in high atorvastatin concentration (2-10 µmol/L).

The cellular uptake of atorvastatin was investigated as a function of incubation time with a constant concentration of atorvastatin (1.5 µmol/L). These experiments indicated that uptake of atorvastatin is more than 2-fold higher in OATP1B1 transfected cells than in mock cells, as shown in figure 3-5. An increasing in cellular uptake of atorvastatin was observed with the increasing of incubation time from 1 minute to 3 minutes. However, the grade of cellular uptake of atorvastatin is not affected by further increasing incubation time up to 40 minutes. The experiments were only performed once at each setting.

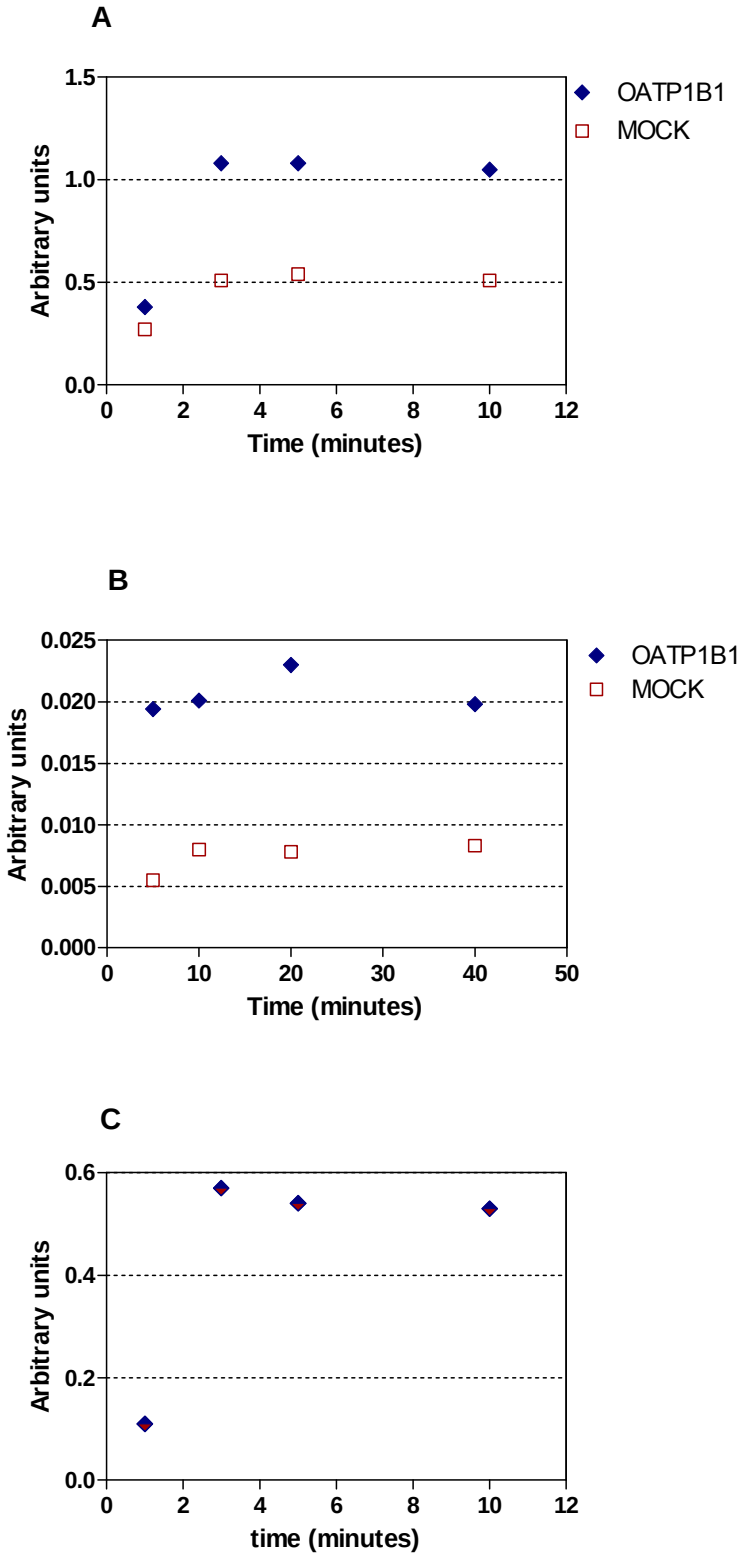


Figure 3-5: Uptake of atorvastatin (1.5 µmol/L) in HEK293 transfected and mock cells as a function of incubation time. Time curves in panel A and B represent the degree of atorvastatin uptake in OATP1B1 transfected and mock cells, in two independent experiments, after incubation with 1.5 µmol/L atorvastatin. The lower part of figure (panel C) displays the differences in uptake of atorvastatin between OATP1B1 transfected and mock cells presented in panel A of this figure. Each point represents the mean value of three parallels. Uptake curves for atorvastatin were obtained on the basis of arbitrary units.

3.4 The inhibitory effect of CsA on cellular uptake of atorvastatin

To investigate the effect of CsA on cellular atorvastatin uptake, both mock and OATP1B1 transfected HEK293 cells were pre-incubated with CsA (0-50 µmol/L) for an hour and then co-incubated with the same concentration of CsA in addition to atorvastatin (3µmol/L) for 5 minutes.

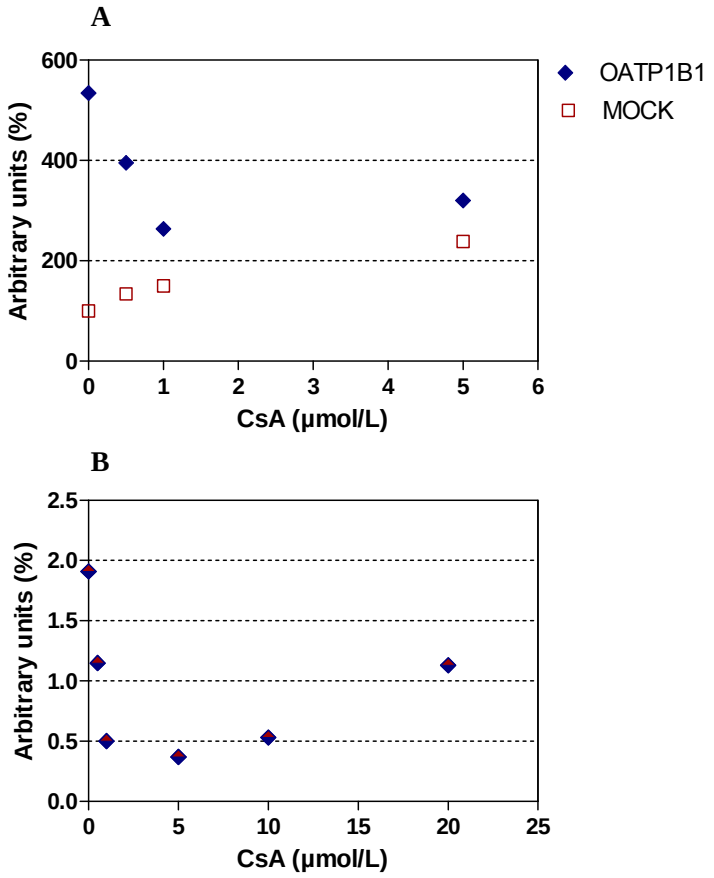


Figure 3-6: Inhibitory effect of CsA on OATP1B1 mediated uptake of atorvastatin in OATP1B1 transfected HEK293 cells. Panel A displays the percentage of arbitrary units when the cells are exposed to CsA at concentration of 0-5 µmol/L. In panel B the Uptake of atorvastatin is given as differences between arbitrary units for OATP1B1 transfected cells and arbitrary units for mock cells of the observations presented in panel A.

Inhibitory experiments by CsA were planned in concentration range of 0-50 µmol/L, but some data have unfortunately been lost during the HPLC-MS/MS analysis, probably due to problems with the sample preparation. The available results are presented in figure 3-6. Pre- and co-incubation with CsA in different concentration contributes to a significant reduction in intracellular atorvastatin concentration in OATP1B1 transfected HEK293 cells with no effect in mock cells. The atorvastatin levels were reduced to a lesser degree with higher

concentration of CsA (>2 µmol/L). The experiments were only performed once at each setting.

3.5 The inhibitory effect of Tac on cellular uptake of atorvastatin

To investigate the effect of Tac on cellular atorvastatin uptake, both mock and OATP1B1 transfected HEK293 cells were pre-incubated with Tac (0-10 µmol/L) for an hour and then co-incubated with the same concentration of Tac in addition to atorvastatin (3 µmol/L) for 5 minutes.

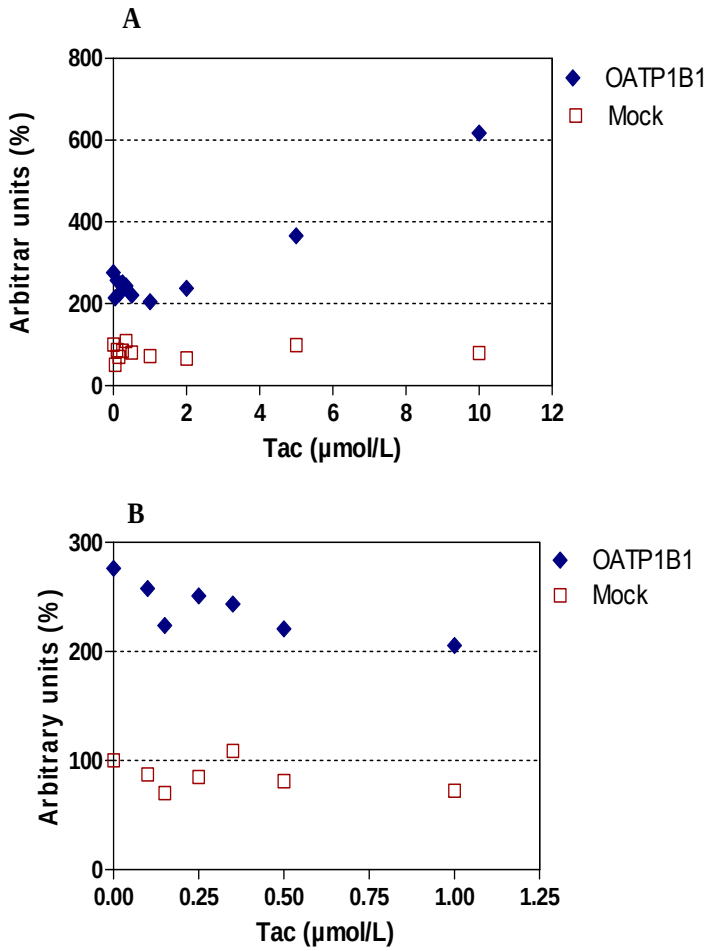


Figure 3-7: The effect of Tac on OATP1B1 mediated uptake of atorvastatin in OATP1B1 transfected HEK293 cells is shown in panel A. The lower panel (B) displays the results more detailed for Tac values in the range 0-1 µmol/L. Uptake of atorvastatin is given as percentage of arbitrary units.

Inhibitory experiments by Tac were planned in concentration range of 0-10 μmol/L, but some data of the only experiment performed was unsuccessful with regards to the HPLC-MS/MS analysis. The available results are presented in figure 3-7. Pre- and co-incubation with low concentrations of Tac (0.1-1 μmol/L) resulted in only a slightly reduction of intracellular atorvastatin concentration. However, incubation with higher concentration of Tac resulted in increasing of atorvastatin concentration, especially in OATP1B1 transfected cells.

4. DISCUSSION

In kidney transplantation patients undergoing CsA treatment, but not Tac, the plasma concentrations of atorvastatin are increased [17, 138, 139] due to interaction between the two drugs. In the present thesis the effect of CsA, compared with Tac, on the OATP1B1 mediated uptake of atorvastatin was investigated to clarify the mechanism underlying this interaction.

4.1 Methodological consideration

4.1.1 Studies of OATP1B1 expression

To verify whether the HEK293 cells expressed OATP1B1, both mRNA and protein analysis were performed. Two different antibodies against OATP1B1 with different concentrations were tested in order to analyse protein expression. However, no specific and satisfactory band for OATP1B1 protein was shown, indicating that these antibodies are not specific enough to OATP1B1 and not suitable for Western blot analysis. The protein bands that did appear in some films were too weak, probably due to that the primary antibody was washed out during the washing-step. Another possible explanation might be that the epitop recognized by the antibodies was destroyed due to protein denaturation during sample preparation. Film developed in Western analysis of the proteins expressed in the OATP1B1 transfected HEK293 cells (figure 3-2) showed weak bands with addition of the Human Hepatic OATP2 Progen antibody (dilution 1:6). The size of the bands is slightly larger than references, possibly due to different conformation compared to the references proteins. Another possible explanation to the higher molecular weight might be that the appropriate post-translational modifications of the proteins have not occurred properly, maybe with the targeting signal still being attached to the polypeptide.

In this study, Western blotting did not act as a sufficiently precise method to determined OATP1B1 expression in HEK293 cells; consequently RT-PCR analysis was applied to study gene expression of OATP1B1. However, RT-PCR methods provide no control whether mRNA is functionally translated to protein.

As expected, the results from gene expression analyses indicate a higher expression of OATP1B1 genes in OATP1B1 transfected cells than in mock cells. Addition of sodium butyrate appeared to have a small, but positive effect in the gene expression level of OATP1B1, as expected.

4.1.2 Studies of cellular uptake of atorvastatin

The primary aim of this thesis was to investigate cellular uptake of atorvastatin in OATP1B1 transfected and mock cells, which was determined by measuring intracellular concentration of atorvastatin utilizing an HPLC-MS/MS method. To ensure the measured atorvastatin values to likely reflect the proper intracellular concentration, the cells were washed properly with PBS buffer twice before harvesting of the cells. However, there is some hesitation whether these concentrations actually represents intracellular atorvastatin or basically atorvastatin associated with cells. The sample preparation process could be improved to ensure measuring concentration to likely be representative of the intracellular concentration of atorvastatin.

Sodium butyrate was added to the HEK293 cells in order to increase gene expression of OATP1B1. Sodium butyrate has however previously been shown to act as an inducer of de novo P-gp in rats [145]. It is possible that an induction effect of sodium butyrate on P-gp in our cells contribute to masking of the OATP1B1 effects (discussed below, section 4.2.2). In future studies, one should control the sodium butyrate effect by also performing atorvastatin cellular uptake experiments in HEK293 cells in the absence of sodium butyrate.

4.1.3 Cellprotein content quantification

The concentration of total cell protein in each well was determined by Bio-Rad Protein Assay in order to correct for variation in amount of cells in each well. The results from the protein quantification did not show to be reproducible in all instance and showed high variability within the parallels. Therefore it was decided not to include the protein quantification data and be satisfied with the cells being seeded into experiment wells at same densities ($5.0x10^5$), and incubated in the identical condition and environment. However, it is considered as a methodological weakness and should be further investigated in future experiments.

4.1.4 Atorvastatin concentration analysis

The sample preparation technique used to measure the atorvastatin concentration in HPLC-MS/MS method in this study is not optimal. In some cases the MS/MS signals have been disrupted probably by some residual compounds from the samples. This affected mainly the MS/MS signals of atorvastatin acid, with only minor effect on the lactone form. In future research, solid-phase extraction might improve the sample preparation and exclude interfering substances which disrupt the MS/MS signals.

The MS/MS signal of methaqualone, previously used as the I.S. in this method, was somewhat variable over time. The retention time of the I.S. should ideally be close to the retention time of atorvastatin. Therefore we decided to use deuterium labelled atorvastatin (Atorvastatin d_5) in addition to methaqualone. The results presented in this study is based on relative MS/MS signal of atorvastatin to MS/MS signal of deuterium labelled atorvastatin (d_5) in order to eliminate the uncertainly around variable value of methaqualone as a I.S.

4.2 Studies of cellular uptake of atorvastatin and the inhibitory effect of CsA and Tac on atorvastatin uptake

4.2.1 OATPP1B1 mediated atorvastatin uptake

Cellular uptake of atorvastatin was observed to a greater degree in OATP1B1 transfected cells than in mock cells. Short incubation time (5 minutes) was selected to minimize potentially unspecific cellular uptake of atorvastatin (to be able to study the true OATP1B1 effect). The observed differences in uptake degree indicate that atorvastatin uptake is at least in part mediated by OATP1B1. This is in accordance with previous *in vitro* studies for atorvastatin [44, 52, 125], and also for other statins [52, 68, 69, 146].

The cellular uptake of atorvastatin in OATP1B1 and mock cells with diverse incubation times were also investigated. Cellular uptake was not observed to alter largely with increasing incubation times from 3 minutes up to 40 minutes at the used concentration of atorvastatin (1.5 μmol/L). This indicates that the uptake of atorvastatin in these cells mainly is mediate by transporters. For further transport investigations, it will perhaps be useful to

study cellular uptake of atorvastatin using different concentration of atorvastatin to elucidate if the concentration of atorvastatin may be of importance to a time curve course.

4.2.2 Inhibitory effect of CsA

Studies of the effect of CsA on cellular uptake of atorvastatin were carried out in order to investigate whether the pharmacokinetic interaction between CsA and atorvastatin is mediated via OATP1B1 transport as speculated upon previously [5, 17, 30, 52, 69, 138, 139]. Considerably inhibition of cellular uptake of atorvastatin was observed in the presence of CsA (especially in the lower concentration range) in OATP1B1 transfected cells. However, the effect of OATP1B1 mediated atorvastatin uptake seems to be affected by other effects at higher concentration of CsA, resulting in elevation of intracellular atorvastatin concentrations. CsA has previously been shown to be an inhibitor of P-gp [35-37], so this might partly explain the observed effect at higher CsA concentration. The reason for why inhibition of P-gp may occur at a greater extent with increasing CsA concentration could be explained by the differences in the value of the equilibrium constant for CsA binding (K_i) to P-gp and OATP1B1. The K_i value for CsA on P-gp and OATP1B1 is reported to be 6.1 µmol/L and 0.24-0.5 µmol/L, respectively [62, 69, 147]. For this reason the inhibitory effect on P-gp will appear first at higher CsA concentrations as compared with OATP1B1 and it would lead to a diminished effect of uptake inhibition of CsA in this model.

Another conceivable explanation could be that the amount of CsA which enter the cells is not high enough for inhibition of efflux transporters until the upper concentration range of CsA is reached. However, the co-incubation time with CsA in these experiments was very short (5 minutes), which theoretically would result in less transport of CsA into the cells and therefore less CsA mediated blockade of efflux transporters like P-gp. A possible improvement in the model would be a reduction in pre-incubation time as well, in order to impediment the transport of CsA into the cells to a greater extent.

Inhibitory experiments by CsA were planned in concentration range of 0-50 µmol/L, but some data have unfortunately been lost during the HPLC-MS/MS analysis. Further investigations in the full concentration range needs therefore to be carried out in order to confirm these findings.

4.2.3 Inhibitory effect of Tac

The inhibitory effect of Tac on OATP1B1 mediated atorvastatin uptake was also investigated in order to study the interaction between atorvastatin and Tac, and to indicate if a possible interaction on OATP1B1 transport will be present when cells are exposed to Tac in molar doses equivalent to CsA doses, but not in clinical relevant doses. No clear inhibition of cellular uptake of atorvastatin was observed in the presence of Tac particularly in OATP1B1 transfected cells, at least not in the lower concentration range. A significant increase in atorvastatin uptake was observed with higher concentration of Tac. This might partly be due to inhibition of efflux transporters such as P-gp by Tac, which is reported to be an inhibitor of this transport [35, 37]. The exact concentration range needs however further optimization in order to make it easier to study the true OATP1B1 effect. These higher concentrations were however not optimally analysed due to technical problems with the HPLC-MS/MS and further investigations, especially at the higher concentrations, are needed.

4.3 CsA versus Tac effects on atorvastatin pharmacokinetic

Previous studies have demonstrated a several-fold increase in systemic exposure of atorvastatin in solid organ transplants recipients by concomitant administration of CsA, a potent OATP1B1 inhibitor and a moderate inhibitor of P-gp, as part of their immunosuppressive therapy [17, 89, 138, 139]. The increased systemic exposure of atorvastatin in renal transplant recipients might be explained by reduced clearance induced by wither inhibition of hepatic uptake transporters (OATP1B1) and/or CYP3A4 metabolism or increased bioavailability via inhibition of biliary or intestinal efflux transporters (P-gp and MRP2) and/or CYP3A4 metabolism. Several studies indicate that atorvastatin is a substrate of MRP2, OATP1B1, P-gp and CYP3A4 [30, 44, 52] and CsA have been shown to be an inhibitor of these transporters and enzymes [36, 45, 46, 62, 69, 146]. Inhibition of hepatic uptake of atorvastatin leads to a decrease in the distribution volume and clearance while terminal half life will remain unchanged as long as these changes are balanced. In previous clinical studies lack of corresponding increase in lipid-lowering effect, despised increased systemic exposure of atorvastatin in CsA treated patients have been shown. All these aspects

provide support for the hypothesis of the presence of a pharmacokinetic interaction based on OATP1B1 transporters between atorvastatin and CsA.

A number of studies indicate that Tac, in contrast to CsA, is a less potent inhibitor of OATP1B1-mediated uptake of substrates into the hepatocytes [62, 89, 138]. These studies suggest that the inhibitory effect of Tac on OATP1B1 may take place first at high molar concentration, while interactions in clinically relevant dosage are not of current interest due to its high K_i value for OATP1B1.

There are two main observations in the present study, both in line with the previously published data. The inhibitory experiments on cellular uptake of atorvastatin mediated by OATP1B1 indicates that CsA yields a noticeable reduction in intracellular concentration of atorvastatin in the OATP1B1 transfected cells, while Tac dose not alter the OATP1B1-mediated atorvastatin uptake, at least not in the lower concentration range.

The results presented in this study may likely certify that the previously observed clinically interaction between CsA and atorvastatin may at least partially be as a result of the inhibitory effect of CsA on OATP1B1-mediated uptake of atorvastatin into the hepatocytes.

4.4 Future considerations

Repeated experiments are necessary in order to verify the inhibitory effect of CsA and Tac on OATP1B1-mediated atorvastatin cellular uptake. The exact concentration range of atorvastatin, CsA and Tac may needs to be closer determined to being able to confirm the findings on this study.

It will be of value to assess whether mRNA of OATP1B1 is functionally translated to protein since the presence of OATP1B1 transporter appears to be the main reason for the differences in atorvastatin uptake between OATP1B1 and mock cells observed in this study. In order to investigate this should protein quantification of OATP1B1 transporters in HEK293 cells be performed using specific antibodies.

The sample preparation techniques as well as analysis technique need to be optimized in order to exclude interfering substances which could disrupt the MS/MS signals. This might be of importance for precisely determining the intracellular concentration of atorvastatin.

In vitro studies show that the acid form of atorvastatin exhibits more potent inhibition toward OATP1B1 compared to the lactone form [44]. This could suggest that the acid form is a better substrate for OATP1B1 compared with the lactone form. In order to perform this comparison, uptake and inhibitory experiments for both acid and lactone form should usefully be carried out in future.

Investigation of the atorvastatin metabolism could be of importance since both metabolites of atorvastatin are active. Further experiments could be carried out in a cell model with co-over vector expression of CYP3A4 and OATP1B1 in order to get the full picture and generally determined other important aspect that should be addressed in the future.

5. CONCLUSION

The present study demonstrates that atorvastatin is transported via OATP1B1, CsA was also shown to be a potent inhibitor of OATP1B1-mediated atorvastatin uptake. This is in contrast to Tac, which did not inhibit OATP1B1-mediated transport of atorvastatin at low, clinically relevant concentrations. Based on these findings it is likely that the interaction between CsA and atorvastatin, observed in previous clinical studies, might partly be due to CsA inhibition of OATP1B1-mediated uptake of atorvastatin. This study provides a starting point to further investigations in order to verify these findings.

6. REFERENCES

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7. APPENDIX

7.1 CHEMICALS

Chemicals	**Manufacturer**
Acetonitrile, HPLC grade	Merk, Darmstadt, Germany
Acrylamid/Bis Solution 30%	BioRad laboratories, 2000 Alfred Noble, Hercules, USA
Agarose, electrophoresis grade	Life Technologies, Paisley, UK
Agilent Total RNA Isolation Mini Kit	Agilent Technologies, Palo Alto, California, USA
Alcohol, absolute Prima	Arcus Kjemi AS, Oslo, Norway
AmpliTaq Gold®	Applied Biosystems, Roche Molecular systems Inc, Branchburg, New Jersey, USA
Atorvastatin	Parke-Davis, Michigan, USA
Bio-Rad Protein Assay Dye Reagent Concentrate	Bio-Rad Laboratories Inc, California, USA
BSA 3%, Sigma no. A-9418	Sigma-Aldrich, St. Louis, Missouri, USA
Carbondioxide gas	AGA Progas A/S, Oslo, Norway
Cyclosporin A	Sigma-Aldrich, St. Louis, Missouri, USA
Deionised water	Easypure UV, Branstead, Iowa, USA
Distilled water	Aquatron A4S, Bibby Science Products SA, France
DMEM	Sigma-Aldrich, St. Louis, Missouri, USA
FBS	Gibco, Paisley, UK
Formic acid, analytical grade	Merck, Darmstadt, Germany
G418 disulphate	Sigma,-Aldrich, Schnelldorf, Germany
GeneAmp®	Applied Biosystems, Roche Molecular

	systems Inc, Branchburg, New Jersey, USA
Gentamycin (10 mg/mL)	Life Technologies, A/S E. Pedersen & Sønn, Oslo, Norway
Goat Anti-Mouse IgG (H+L)	BioRad laboratories, 2000 Alfred Noble, Hercules, USA
Goat Anti-Rabbit IgG	BioRad laboratories, 2000 Alfred Noble, Hercules, USA
Hanks' Balanced Salt Solution	PAA Laboratories GmbH, Pasching, Austria
Human Hepatic OATP2 IgM Antibody	Biotechnik, Heidelberg
KH_2PO_4, analytical grade	Merck, Darmstadt, Germany
Methanol, HPLC grade	Merck, Darmstadt, Germany
Methaqualone	Sigma-Aldrich, St. Louis, Missouri, USA
$MgSO_4.7H_2O$, analytical grade	Merck, Darmstadt, Germany
Na_2HPO_4, analytical grade	Chemi-Teknik A/S, Oslo,Norway
Nitrogen gas	AGA Progas A/S, Oslo, Norway
OATP2 (H-60) Antibody	Santa Cruz Biotechnology, INC, California
P/E (0.1 mM), Biofluids no. 353	Biofluids, Rockville, California, USA
Penicillin	Gibco, Paisley, UK
Poly-D-lysine	Sigma-Aldrich, St. Louis, Missouri, USA
Precision Plus Protein ^{TM} Dual Colour Standards	BioRad laboratories, 2000 Alfred Noble, Hercules, USA
Primers	Invitrogen, Carlsbad, California, USA
Sodium butyrate	Sigma-Aldrich, St. Louis, Missouri, USA
Streptomycin	Gibco, Paisley, UK
TaqMan® Reverse Transcription Reagents	Applied Biosystems, Roche Molecular systems Inc, Branchburg, New Jersey, USA
TEMED, N,N,N,N-Tetra-Methyl-Ethylenediamine	BioRad laboratories, 2000 Alfred Noble, Hercules, USA

Trypan Blue	Sigma-Aldrich, St. Louis, Missouri, USA
Trypsin-EDTA (1x), 0.05% trypsin, 0.53 mM EDTA.4 Na	Gibco, Paisely, UK
Tween® 20	BioRad laboratories, 2000 Alfred Noble, Hercules, USA

7.2 EQUIPMENT

Instruments	**Manufacturer**
Avanti ™ J-25 Centrifuge	Beckman Coulter Inc, Fullerton, California, USA
Biofuge Fresco Centrifuge	Heraus instruments, Heraus, Germany
Büker counting chamber	Assistant, Sontheim, Germany
C_{18} analytical column (Omnisphere C_{18}, 3 µm, 30x2 mm)	Varian, Lake forest, California, USA
ChromoSepGaurd column (3 µm, 10x2mm)	Varian, Lake Forest, California, USA
DU® 530 Life Science UV/Vis Spectrophotometri	Beckman Coulter Inc, Fullerton, California, USA
Electrophoresis power supply – EPS 600	Pharmacia Biotech AB, Uppsale, Sweden
Finnigan LCQ^{DUO} ion trap MS detector	Instruments Teknikk AS, Østerås, Norway
GeneApm PCR System 9700	PE Applied Biosystems, Foster City, California, USA
Holten LaminAir	Medinor, Oslo, Norway
Innova TM 4330 refrigerated Incubator Shaker	New Brunswick Scientific, Edison, New Jersey, USA
Kubota 2010 Centrifuge	Medinor, Oslo, Norway
NUAIRE™ US Autoflow CO_2 Water-Jacketed Incubator	NuAire, Plymouth, Minnesota, USA

Reichert Microscope	Reichert, Austria
Semi-Dry Blotters	Hoffer Scientific instruments, San Francisco, California
Agilent Technologies 1200 series autosampler	Agilent Technologies, Santa Clara, California, USA
Agilent Technologies 1200 series quarterary pump	Agilent Technologies, Santa Clara, California, USA
Agilent Technologies 6310 LC/MS ion trap	Agilent Technologies, Santa Clara, California, USA
The Belly Dancer	Storvall, Life Science, INC Greensboro, NC, USA
Uiversal 32R Centrifuge	Hettich Zentrifuge, Tuttlingen, Germany

7.3 CLINICAL PHARMACOKINETICS OF ATORVASTATIN

Table 7-1: Clinical pharmacokinetics of Atorvastatin [128, 148, 149]

Parameters	*Atorvastatin*
T_{max} (h)	2-3
C_{max} (ng/mL)	27-66
Absolute oral bioavailability (%)	12
Lipophilicity	Yes
Protein binding (%)	80-90
Metabolism	CYP3A4
Metabolites	Active
Transporter protein substrate	Yes
$T_{½}$ (h)	15-30
Urinary excretion (%)	1
Fecal excretion (%)	70
CL_{tot} (mL/min)	625
E_H	0.4
Log D (pH 4)	4.2
Solubility (mg/ml at pH6)	1.23
Absorbance (6.0-6.5)	Complete

7.4 Antibodies used in Western blotting

Table 7-2: Primary and secondary antibodies used in Western blotting

Primary antibodies (AB)	*Dilution of primary AB*	*Secondary AB*	*Dilution of secondary AB*	*Gel %*	*Exposure minutes*
Human Hepatic OATP2 mouse monocolonal	1:6	Goat anti-mouse IgG (H+L)	1:1500	12	5/30
Human Hepatic OATP2 mouse monocolonal	1:10	Goat anti-mouse IgG (H+L)	1:1500	12	5/30
Human Hepatic OATP2 mouse monocolonal	1:100	Goat anti-mouse IgG (H+L)	1:1500	10	5/30
Human Hepatic OATP2 mouse monocolonal	1:500	Goat anti-mouse IgG (H+L)	1:1500	10	5/30
Human Hepatic OATP2 mouse monocolonal	1:5000	Goat anti-mouse IgG (H+L)	1:1500	10	5/30
OATP2 (H-60) Antibody	1:200	Goat anti-rabbit IgG	1:3000	12	5/30
OATP2 (H-60)	1:500	Goat anti-rabbit IgG	1:3000	12	5/30
OATP2 (H-60) Antibody	1:500	Goat anti-rabbit IgG	1:3000	10	5/30
OATP2 (H-60) Antibody	1:1000	Goat anti-rabbit IgG	1:3000	10	5/30

7.5 RECIPES

7.5.1 Solution for culturing of HEK293 cells

Growth medium	
DMEM, high glucose (4.5 g/L)	1000mL
FBS	100 mL
Penicillin	$1x10^5$ U
Streptomycin	100 mg
G418	600 mg

Coating medium for 6-well plates	
Poly-D-lysine 0.5 mg/mL	0.353 mL
Distilled H_2O	17.65 mL

Trypsin-EDTA diluted with HBSS (1:1, v/v)	
Trypsin-EDTA (1x)	25 mL
HBSS	25 mL

Trypan blue 0.4%	
Trypan blue	0.4 g
Solution E	Ad 100 mL

Solution E	
NaCl	8.5 g
KCl	0.5 g
KH_2PO_4	50 mg
Na_2HPO_4	60 mg
$MgSO_4 \cdot 7\ H_2O$	0.2 g
Hepes	4.8 g
$CaCl_2 \cdot 2H_2O$	0.15 g
Distilled H_2O	Ad 1000 mL

7.5.2 Solution for PCR analysis

10xPBS, sodium citrate buffer

$NaH_2PO_4 \cdot H_2O$	1.57 g
$Na_2H_2PO_4 \cdot 2H_2O$	9.8 g
$Na_3C_6H_5O_7 \cdot 2H_2O$	60 g
NaCl	81.8 g
Distilled H_2O	Ad 1000 mL, pH 7.4
	Autoclave

1xPBS

10xPBS	10 mL
DEPC-H_2O	Ad 100 mL

DEPC-dH_2O (0.1%)

DEPC	1 mL
Distilled H_2O	Ad 1000 mL
	Autoclave

TagMan® reaction mix used in RT (volumes given per sample)

10xTagMan RT Buffer	2.0 µL
$MgCl_2$ (25 µM)	4.4 µL
dNTP mix (10 mM)	4.0 µL
Random hexamers (50 µM)	1.0 µL
RNase inhibitor (20U/µL)	0.4 µL
Multiscribe Reverse Transcriptase (50 U/µL)	0.5 µL

GeneAmp® reaction mix used in PCR

DEPC-dH_2O	30.6 µL
10xPCR Buffer	5.0 µL
$MgCl_2$ (50 µM)	1.5 µL
dNTP mix (10 mM)	1.0 µL
Primer R	0.8 µL
Primer F	0.8 µL
Taq DNA polymerase (5 U/µL, AmpliTaq Gold)	0.3 µL

7.5.3 Solution for agarose gel electrophoresis

5xTBE, Tris-borate buffer

Tris	54 g
HBr	27.5 g
0.5 M EDTA pH 8.0	20 mL
Distilled H_2O	Ad 1000 mL
	Autoclave

1xTBE

5xTBE	200 mL
Distilled H_2O	Ad 1000 mL

Ethidium bromide (10 mg/mL)

Ethidium bromide	0.2 g
Distilled H_2O	20 ml

Loading dye buffer

Bromophenol blue	0.25 g
Xylene cyanol FF	0.25 g
Ficoll	15 g
Ddistilled H_2O	Ad 1000 mL

DNA standard (0.1 µg/mL),

Ladder, 100 bp, 1 µg/µL	20 µL
Loading dye buffer	33 µL
Distilled H_2O	147 µL

2% agarose gel

Agarose	2 g
5 mg/mL ethidium bromide	5 µL
1xTBE	100 ml

7.5.4 Solution for protein quantification

Protein assay dye reagent

Dye Reagent Concentrate	1 part
Distilled H_2O	2.75 parts
sterile filtrated by use of 0.22 µm Millipore membrane filter	

7.5.5 Solution for studies of cellular uptake of atorvastatin

PBS buffer

NaCl	8 g
KH_2PO_4	0.2 g
$Na_2HPO_4 \cdot 2H_2O$	2.9 g
KCl	0.2 g
	Ad 1000 mL
	pH 7.2-7.4

7.5.6 Solutions for HPLC-MS/MS

Methanoic acid 1 mM

Methanoic acid, concentrate	37.7 µL
Distilled H_2O	Ad 1000 mL

Mobil Phase A

Acetonitrile (AcN)	300 mL
Methanoic acid 1 mM	700 mL

Mobil Phase B

Acetonitrile (AcN)	600 mL
Methanoic acid 1 mM	400 mL

7.5.7 Solutions for Western blotting

10% separating gel	
Distilled H_2O	5.32 mL
1.5 M Tris-HCl pH 8.8	3.25 mL
Monomer	4.33 mL
10% SDS	130 µL
10% ammoniumpersulfate (APS)	65 µL
TEMED	6.5 µL

12% separating gel	
	4.36 mL
1.5 M Tris-HCl pH 8.8	3.25 mL
Monomer	5.20 mL
10% SDS	130 µL
10% ammoniumpersulfate (APS)	65 µL
TEMED	6.5 µL

4% stacking gel	
Distilled H_2O	6.10 mL
0.5 M Tris-HCl pH 6.8	2.50 mL
Monomer	1.30 mL
10% SDS	100 µL
10% ammoniumpersulfate (APS)	50 µL
TEMED	10 µL

Electrophoresis buffer pH 8.3	
0.025 M Tris-HCl	7.5 g
SDS	2.5 g
0.192 M Glycin	35 g
Distilled H_2O	2.5 L

Blotting buffer pH 8.3	
0.025 M Tris-HCl	12 g
SDS 0.02%	0.8 mL
0.192 M Glycin	57.6 g
20% MeOH	800 mL
Distilled H_2O	2.5 L

Sample buffer

0.5 M Tris-HCl pH 6.8	12.5 mL
Glycerol	10 mL
10% SDS	20 mL

2xLaemmLi buffer

Tris-HCl	0.0625 M
SDS	0.1%
Glycerol	15%

1xLaemmLi buffer

2xLaemmLi buffer	50%
Distilled H_2O	50%

T-TBS

Tris, pH 7.5	10 mM
NaCl	100 mM
Tween	0.05%
Distilled H_2O	Ad 100%

Blotto (125 mL/membrane)

Fat-free dry milk	50 g
T-TBS	1 L

Fixation solution

Fix	120 mL
H_2O	280 mL

Developing solution

Developer	120 mL
H_2O	280 mL